MELANOPSIN SIGNALING IN MOUSE IRIS

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Abstract

The pupil size is determined by an antagonistic action between the circumferential sphincter muscle and the radial dilator muscle in the iris. Upon illumination, the pupils in both eyes constrict to a broadly similar extent because of the bilateral reflex pathways through the brain. Despite this widely-accepted picture, it has been reported that, the isolated iris sphincter muscle of sub-primate nocturnal and crepuscular mammals generally shows, surprisingly, a light-induced contraction. In mouse, this local iridic light effect requires the pigment, melanopsin, the localization and signaling mechanism of which remains unclear. Others have suggested that the local light reflex of the isolated mouse iris sphincter results merely from collateral axons of intrinsically-photosensitive retinal ganglion cells (ipRGCs) forming presynaptic terminals on parasympathetic muscarinic innervation of the sphincter muscle cells. Here, we exclude this model by showing that atropine, a muscarinic blocker, eliminated the effect of acetylcholine (ACh) on the isolated mouse sphincter muscle without affecting its light response, indicating that the light-induced contraction of the isolated iris sphincter is independent of muscarinic synaptic transmission. By genetic labeling, we underpinned melanopsin's expression in about 10% of mouse iris-sphincter muscle cells, in which light and acetylcholine appear to signal through shared, albeit not identical pathways. The response of iris sphincter muscle isolated from the conditional $G\alpha_q$ $G\alpha_{11}$ double-knockout line to ACh was practically the same as control, but the muscle's response to a light flash was significantly reduced.

Knockout of genes encoding other members (i.e. *Gα$_{14}$* or *Gα$_{15}$*) from $G_{q/11}$-subfamily did not affect the muscle's response to ACh or light, indicating that melanopsin signals to both $G\alpha_q$ and $G\alpha_{11}$ in the iris muscle cells. Iris sphincter isolated from *Plcβ2*$^{-/-}$ animal also showed defective response to light flashes, but the response to ACh stimulation was largely preserved, suggesting that different sets of PLCβs are expressed in light-responsive versus light-irresponsive sphincter muscle cells. Knockout of *Itpr1* eliminated both light- and ACh-induced muscle contraction, indicating that the response to both stimuli relies on release of Ca^{2+} from the intracellular store.

The overall conclusion from this work is that the light induced muscle contraction is generated from a subpopulation of sphincter muscle cells that express melanopsin. The contractions of the iris sphincter muscle elicited by ACh and by light are via separate signaling pathways, although these pathways converge at the step of actin-myosin interaction triggered by a rise in intracellular Ca^{2+} concentration. This study generated the first molecular and genetic analysis of the iris-based photoreceptors, expanded our knowledge on the mechanism of photoreception mediated by melanopsin.

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1. Introduction

1.1 General structure of the mammalian iris

The iris is a circular structure surrounding the pupil of the eye, responsible for controlling the amount of light entering the eye interior. It consists of three layers[1]. The most anterior layer is a pigmented fibrovascular stroma, followed by a smooth muscle layer (including the sphincter and the dilator muscle), and the innermost layer is the iris pigmented epithelium (IPE). The pigment in both the iris stroma and IPE prevents light from passing through the iris into the eye, restraining it to the pupil. The shape of the pupil varies in different species. In rodent and primate, the pupil is circular in both dilated and constricted conditions. Ambush predator has vertically elongated pupil when contracted. In grazing animal, however, the constricted pupil is horizontally elongated[2].

The size of the pupil is set by an antagonistic action between the sphincter muscle and the dilator muscle[1]. The sphincter muscle of the iris is annular band of smooth muscle running parallel to the edge of the pupil, which contract the pupil in a circular motion. The dilator muscle pulls the iris radially to enlarge the pupil.

1.2 Musculature: sphincter pupillae and dilator pupillae

The sphincter muscle lies closer to the pupil, occupying a fraction of the radial width of the iris (known as the sphincter pupillae). The muscle cells are in close

apposition to each other, with extremely reduced intercellular space. The size of the muscle cells was estimated to be similar to those of visceral muscle cells (3-5 μm in diameter in the region of their nuclei, and 150-250 μm long)[3,4]. The transverse section of the muscle displays caveolae and gap junction[1,3,5]. In guinea pig, it was estimated that 0.75% of the cell membrane is occupied by gap junctions, considerably higher than the other types of smooth muscle in the same species[1].

The dilator muscle (or dilator pupillae) lies closer to the iris pigmented epithelium than to the anterior part of iris. These cells are elongated and run radially from the edge of sphincter pupillae to the ciliary body. In rat, it was reported that there was no overlap between the two muscle types[6], although some overlap was reported by other authors[7]. In contrast to the sphincter muscle, the dilator muscle cells do not form a continuous layer. The muscle cells are connect to each other and to the epithelial cells by gap junctions[1].

1.3 Innervation and control of the mammalian iris smooth muscle

Nerve fibers, originating from ciliary ganglion and superior cervical ganglion, are extremely abundant in the sphincter pupillae[1,8–12]. The fibers of the above origins are unmyelinated, often run within the same nerve bundles[1]. The nerve in the sphincter pupillae forms a series of swellings along the terminal portion of the axon (known as axon varicosities), where the neurotransmitter-containing vesicles can be found. The axon varicosities lie in close vicinity to the sphincter muscle, often in contact with two or three cells. In contrast to the situation in the

skeletal muscle, the nerve endings in sphincter pupillae do not usually show junctional specializations[1,4]. Therefore, vesicles released from the axon terminal diffuse onto several muscle cells. On this basis, it is not surprising that a smooth muscle cell may receive a complex of chemical signals from the nervous system, both positive and negative in causing its contraction[4].

The cholinergic ciliary ganglion provides motor innervation to the iris sphincter. Each neuron issues one single axon, which enters the iris through the ciliary body and gives rise to extensive peripheral arborization, innervating not only sphincter pupillae, but also dilator pupillae[12]. The concept of dual innervation of the two types of muscles of the mammalian iris has been well established[1,8,10]. Adrenergic fiber originating from superior cervical ganglion also could be identified in both of the sphincter pupillae and dilator pupillae. Furthermore, varicosities of adrenergic and cholinergic often occur side by side within both types of muscles[11].

Sensory fibers originating from the trigeminal ganglion are also present in the mammalian iris, including all myelinated fibers and a few of the unmyelinated fibers[1,13–16]. The nerve fibers of the trigeminal ganglion origin are also immunopositive for substance P, calcitonin gene-related peptide (CGRP) and sensitive to capsaicin treatment[14–16].

The forth ganglion that innervates the mammalian iris is pterygopalatine ganglion[1]. The neural fibers of this origin may contain vasoactive intestinal polypeptide (VIP), because removal of ciliary ganglion, superior cervical ganglion,

and capsaicin treatment were all ineffective in removing the VIP-immunopositive iris nerves[1].

1.4 Receptors in iris sphincter and dilator pupillae and pupillary size regulation

The well-accepted scheme for pupillary size regulation is that the parasympathetic tone increases as the amount of light reaching the retina increases, whereas the sympathetic tone decreases, resulting in the contraction of the sphincter muscle by acetylcholine (ACh) released from the cholinergic fibers, and relaxation of the dilator muscle. In the course of pupil constriction, the retinal light stimulation decreases, therefore the neuromuscular responses are reversed: the dilator muscle is activated by norepinephrine (NE) released from the sympathetic nerves and the sphincter muscle relaxes.

This generally-accepted pupillary size regulation, however, is oversimplified, as indicated by many histological and functional studies on the double reciprocal innervation by cholinergic and adrenergic nerve fibers to both iris muscles[1,8,10]. To fully understand the autonomic regulation of pupil size, the receptors on each type of iris muscle need to be reviewed.

1.4.1 Muscarinic receptors in sphincter and dilator pupillae

ACh receptors in the iris smooth muscle belong to the muscarinic types. There are five types of muscarinic receptors, designated M_1-M_5AChR[17]. The precise nature of muscarinic receptors mediating the contraction of iris sphincter muscle has been deduced from both biochemical and pharmacological studies from

different animal species. All five types of muscarinic receptors were identified in the human eye by immunoprecipitation, of which M_3AChR predominates in the sphincter muscle[18]. *In situ* hybridization revealed that bovine iris sphincter also contains predominantly the M_3AChR[19]. Functional studies using knockout mouse lines have lent further support to the role of M_3AChR in activating iris sphincter contraction[20,21]. The still incomplete removal of pupillary reflex in mice lacking M_3AChR suggests the involvement of other subtypes of muscarinic receptors[21]. The antagonist profile of muscarinic receptors in rabbit iris sphincter is inconsistent with M_1-M_4AChR[22], postulating the role of M_5AChR in the sphincter contraction.

Muscarinic receptors are also present in the dilator pupillae. It has been known that application of a low dose of ACh (≤1 μM) results in a relaxation of the dilator preparations isolated from cat[23], rat[6], cattle[24], pig[25] and human[9]. The ACh-induced dilator muscle relaxation is blocked by atropine, possibly through the antagonization of the muscarinic inhibition of NE overflow (due to the basal release of ACh in the iris)[26]. The observation of parasympathetic denervation that also abolishes ACh-induced relaxation of dilator muscle further indicates the existence of prejunctional muscarinic receptors[27]. These prejunctional receptors in rat[28] and rabbit[29] are of the M_2-subtype. Therefore, the relaxation effect of ACh on the iris dilator is likely from the presynaptic inhibition of NE release in the dilator pupillae.

The action of ACh on rat iris dilator muscle is complex, with a relaxation to low dose of ACh (≤1 μM), but a contraction to higher dosage (≥ 1uM). By using antagonists against different muscarinic receptor subtypes (M_1-M_5), Masuda and colleagues[30] demonstrated that the muscarinic receptor mediating the contraction of dilator appears to be M_3-subtype. The ACh-induced contraction of rabbit dilator muscle was reported by Persson and Sonmark[31]. The contraction of the dilator in response to ACh is ~3 times smaller compared with that induced by 1-noradrenaline or 1-adrenaline. Atropine and alpha-adrenergic receptor antagonist blocked the ACh and 1-adrenaline induced dilator muscle contraction, respectively. These results suggest that the rabbit iris dilator muscle contains both muscarinic and adrenergic receptors, although the muscle contraction generated from the adrenergic receptor is dominant.

1.4.2 Intracellular signaling by muscarinic receptors

It has been generally accepted that M_1, M_3, M_5-subtypes of muscarinic receptors signal via pertussis toxin insensitive $G_{q/11}$ family of G-protein to phospholipase C-β (PLCβ), thus mobilizing intracellular calcium and causing muscle contraction[32,33]. M_2 and M_4-subtype of receptors preferentially activate G_i family of G-proteins, leading to decrease in cAMP production and Ca^{2+} conductance[32,33].

The important role of Ca^{2+} in the activation of smooth muscle contraction, including iris sphincter muscle, has been well established. The activated PLCβ produces two second messengers, inositol triphosphate (IP_3) and diacylglycerol (DAG) through the hydrolysis of phosphatidylinositol 4,5-bisphosphate

(PIP_2)[1,34,35]. In smooth muscle, Ca^{2+} could enter the cell via voltage gated ion channels on the plasma membrane, or be mobilized from intracellular store by second messengers[35]. The observation of loss of contractile response to ACh after Ca^{2+} depletion from the bath solution[36] could be explained by either an exclusive dependency on the entry of extracellular Ca^{2+}, or a rapid loss of stored Ca^{2+}. In light of the differential inhibitory effect of D-600 (calcium channel blocker) on ACh and K^+-depolarization induced Ca^{2+} increase[36], and lack of membrane potential change in response to ACh, the involvement of intracellular store may prevail in causing the contraction of iris sphincter muscle.

In iris sphincter muscle, rising in intracellular Ca^{2+} in response to ACh activates calmodulin, which then in turn activate myosin light chain kinase (MLCK). Phosphorylation of myosin light chain by MLCK allows it to cross-bridge with actin, thus generating serial conformational binding cycles and muscle contraction[34].

1.4.3 Adrenergic receptors in sphincter and dilator pupillae

Adrenergic receptors can be divided into α- and β-receptors based on Ahlquist's classification[37]. Each adrenergic major type can be further divided into two or more additional subtypes. α_1-receptors are excitatory in terms of causing muscle contraction, because they signal via $G_{q/11}$-PLC pathway to mobilize intracellular calcium[38]. β-receptors, on the other hand, are inhibitory as they activate Gs[39] to stimulate the production of cAMP (smooth muscle relaxant).

The rabbit iris sphincter muscle contains numerous muscarinic receptors, thus producing strong contraction in response to ACh. However, there are also β-adrenergic receptors present in the sphincter muscle[31], which was demonstrated by the fact of 50% inhibition of the maximal sphincter muscle contraction in the presence of β-adrenergic specific agonist[31]. In cat iris, low dose of NE often induces contraction of the sphincter muscle[23]. In contrast, response to high dosage of NE are variable, including contraction, relaxation, and biphasic response consisting of a contraction followed by relaxation[40]. The mixed behavior might reflect the different percentage of excitatory α-receptor and inhibitory β-receptor in the sphincter muscle[1]. Dilator muscle, for instance in rabbit, has numerous alpha-adrenergic receptors, but only a few beta-adrenergic receptors and muscarinic receptors. Therefore, the dilator muscle produces a strong contraction to adrenergic stimulation.

To sum up, the distribution and function of different types of receptors agree with the dual innervation of cholinergic and adrenergic fibers into both of iris muscles, and the synergistic function of sphincter and dilator muscle in pupil size control. Adrenergic nerve stimulation may cause a contraction of dilator muscle and relaxation of sphincter muscle, thus pupil dilation. Cholinergic nerve stimulation, on the other hand, may contract both muscles. However, ACh-induced contraction of dilator is negligible comparing with the contraction of sphincter muscle, and of minimal effect in affecting the overall size of the pupil.

1.5 Photosensitivity of the iris muscle cells

In addition to the neural regulation of pupillary reflex, it has long been known that the isolated irises from a wide variety of vertebrates, from fish to mammal, contract *in vitro* when light is shone on them[4,41–45]. Because of the iris is isolated from the rest of the brain, it was generally believed that the direct contractile response to light (also known as photomechanical response, or PMR) originating from the sphincter muscle cells that possibly express a photoreceptive protein[4].

1.5.1 Discovery of the photomechanical response (PMR)

In neurally driven consensual reflex, both eyes constrict in response to illumination of one eye. Conversely, experiments demonstrating the presence of PMR in early days often use a local stimulation focusing on the iris alone, and ask if the contraction of the sphincter pupillae could be elicited *in vivo*. This method, unfortunately, is not very sensitive, and consequently, PMR has been confused as a general phenomenon[4]. An isolated preparation containing the anterior chamber of the eye or the iris sphincter pupillae was then used as a more reliable strategy to identify PMR in different species.

The PMR in frog has been studied most extensively, and it was also reported that it exists in virtually all amphibians examined[4]. To exclude the possibility of pupillary constriction resulted from any light scattering on to the retina, Steinach[46] demonstrated that PMR persists in the isolated frog iris. He also investigated the location of the photosensitive sphincter cells by using a light spot to stimulate different regions in the isolated frog iris, and pointed out that the light spot focusing on the internal edge of the iris is most effective in inducing muscle

contraction. Furthermore, the contraction generated from the locally stimulated area spreads to the other side of the iris.

To identify the photoresponsive protein in the frog iris, Magnus stimulated the isolated frog iris by a spectrum of light emitted from a prism, and claimed that the largest response was obtained by green light stimulation, suggesting that the underlying pigment is rhodopsin[47]. However, he also reported that the PMR was affected by atropine, suggesting that the contraction of iris sphincter was not due to a direct effect of light on muscle fibers, but was mediated by way of neural element within the iris. This is not in agreement with other *in vivo* experiments showing that a lethal dosage of atropine did not abolish PMR[48]. The results described by Magnus was possibly due to the irreversible damage to the muscle during experiment as the response to both light and electricity decreased together[42,49].

Barr and Alpern provided more quantitative analysis on the PMR in frog iris[42]. The action spectrum of the isolated frog iris was generated by plotting reciprocal of quantal number required for producing the same amplitude of response under the conditions where the magnitude of response is proportional to the number of incidental quantal[42]. The action spectrum was not in a complete agreement with the absorption spectrum of rhodopsin, suggesting that the PMR in frog may not be solely mediated by rhodopsin[42]. The ineffectiveness of adrenergic and cholinergic inhibitors on the PMR ruled out the possibility of NE or ACh in inducing the muscle contraction in response to light stimulation. However, the

possibility remains that the photoreceptive protein, i.e. rhodopsin, resides in non-muscle cells, which activates the contraction of muscle cells by releasing factors that are permeable to the cell membrane[42]. The source of Ca^{2+} in activating muscle contraction was also examined by the authors. When the muscle was placed in Ca^{2+} free solution, the duration for the response amplitude to drop to 50% of the maximum level was about one hour and a half, which is too slow to be considered as a function of removing Ca^{2+} from the extracellular space. Similar to the ACh induced muscle contraction, membrane depolarization is not required in the PMR[42].

1.5.2 PMR in mammals

Bito and Turansky provided the first study of PMR in three rodent species: rat, hamster, and guinea pig[43]. The light response can be demonstrated on the isolated anterior segment of eyes from hamster and, to a much less extent rat, but not guinea pig. The greatest photosensitivity of the isolated hamster iris was observed to light of 440-500 nm. With 1μM atropine, a concentration that is 10 times higher than that to abolish miotic effect (pupil constriction) of carbachol (cholinergic agonist), the PMR was largely preserved in the isolated hamster iris. The light sensitivity is a general and pronounced phenomena in pigmented and albino hamster. However, a considerable animal-to-animal variation exists, possibly due to the use of pupil arear as a readout for the light response.

In 2011, a study by Xue and colleagues identified that the direct response of isolated iris to light is widespread across non-primate mammals by measuring

the force generated from muscle contraction[45]. The animals tested positively for the intrinsic pupillary light reflex (iPLR) are either nocturnal (e.g. mouse, rat, and hamster), or crepuscular (e.g. dog, cat, and rabbit). None of the primates tested, including both nocturnal and diurnal, showed any iPLR. The authors further demonstrated the functional implication of iPLR *in vivo* by isolating the intrinsic response from the consensual reflex through optic nerve transection. The results suggest that the iPLR starts to be activated when the ambient light reaches a level that equals to common room light. Furthermore, the iPLR itself is able to drive pupil constriction to 80-90% to completion. In short, nocturnal/crepuscular non-primate mammals tend to have an iPLR. It is at present not clear what causes the disappearance of iPLR in primate, even though the mRNA of the photoreceptive gene (see 1.5.4) is present in the iris.

1.5.3 The relationship between iPLR and neural control of PLR

The importance of cholinergic stimulation relative to the iPLR has been evaluated in amphibians, whose pupil is primarily closed by the direct light-induced contraction of the sphincter muscle. In lower vertebrates, the iPLR plays more significant role in controlling pupil diameter. In the case of Cyclostomes, whose pupil was reported to be devoid of neural innervation, the only way to activate pupil constriction is via the direct effect of light on the sphincter muscle[4]. In mammals, however, the iPLR is weaker relative to the nerually driven PLR. For example, in rabbit and rat, maximum muscle contraction induced by light is about one tenth of the strength in response to millimolar of ACh. However, the exact ratio between the two needs to be revisited by more quantitative and

reproducible measurements. Furthermore, the iPLR becomes activated under higher light intensities (see also Ref 45), sustaining pupil constriction under the condition of decreased outflow of retinal activity in the course of pupillary reflex.

1.5.4 Light responsive protein in mammalian iris

The action spectrum of the isolated mouse iris fits an A_1 pigment spectrum with a λ_{max} of 480 nm, coincided with the absorption peak of melanopsin, which is the visual pigment that is primarily expressed in a subset of the retinal ganglion cells. Genetically, knockout of *opn4*, which is the gene encoding melanopsin, abolished the muscle's response to light. The potential role of rhodopsin or cryptochromes in the iPLR has been ruled out by examining the muscle's light response using corresponding knockout mouse lines. Rhodopsin mRNA could be detected in the mouse iris, however, its functional significance is not clear.

Melanopsin-expressing ganglion cells (also called intrinsically photosensitive retinal ganglion cells, or ipRGCs) are the third class of photoreceptors in mammalian eyes. These cells, like the conventional ganglion cells, not only receive light information from rods and cones, but also respond directly to light stimulation. Melanopsin is the photoreceptive protein in ipRGCs, and that different types of non-photosensitive cells can be made photoresponsive by introducing melanopsin. The molecular mechanism of how melanopsin transduce light signal has been investigated[45]. In many ways, the vertebrate melanopsins resemble invertebrate-like photopigments, which use phospholipase C-mediated phototransduction cascade. Indeed, melanopsin activates phospholipase Cβ-4

(PLCβ4), leading to the opening of Transient receptor potential channels 6 and 7 (TRP6 and TRP7). The Gα subunit signals between melanopsin and PLCβ4 remains unknown. Pharmacological evidence suggest that it should belong to $G_{q/11}$ subfamily[50]. However, *in vitro* expression suggests the ability of melanopsin to activate G-proteins from multiple subfamilies. In fact, many G-protein coupled receptors are promiscuous, therefore experimental evidence from non-native cell types only reflects activation of whatever signaling components that are available, and may not translate to *in vivo* physiology.

1.5.5 Cellular and Molecular mechanism of the iPLR in mouse

The iPLR persists in an isolated iris preparation, indicating that melanopsin-expression is very likely to be found in the iris sphincter muscle cells, or cell types that are in close vicinity of the smooth muscle. By using a mouse line that express the fluorescent protein tdTomato under the control of melanopsin promotor, Xue and colleagues reported the fluorescent signal in the inner margin of the iris[45]. Melanopsin immunoreactivity also partially colocalized with a smooth muscle marker. However, strictly speaking, because melanopsin immunoreactivity is more widespread than the sphincter muscle region, contraction is not necessarily initiated by light absorbed just in the muscle itself[45].

Recently, two groups[51–54] identified melanopsin-positive neural processes on the edge of the iris. Studies from Rupp and colleagues[51] suggest that they originate from a subset of M1 ipRGCs (which express the highest level of melanopsin), and that the light response of the isolated iris is not from the photosensitive

muscle cells, but rather being mediated by the direct projections from iPRGCs to the iris muscle. The authors proposed that the ipRGCs axonal collaterals travel intraocularly and presynaptically drive the parasympathetic cholinergic terminals innervating the muscle. The fact that *in vivo* application of atropine, which antagonizes the excitatory parasympathetic innervation to the sphincter muscle, abolished the pupil constriction is not inconsistent with the role of cholinergic synaptic transmission in light-induced sphincter muscle contraction. However, application of atropine not only inhibits sphincter muscle, but also increases the NE overflow to the dilator muscle due to the reverse of the inhibitory effect of M_2AChR on the sympathetic nerve terminal (see section **1.4.1**). In short, to ask specifically whether the sphincter muscle is photoresponsive, there is no better preparation than directly measuring the light effect on the isolated mouse iris sphincter.

1.5.6 PMR in birds

The isolated embryonic chicken iris sphincter muscle is also able to contract in response to light stimulation[55,56]. The action spectrum of chicken iris indicates that it is most sensitive to short-wavelength light stimulation, consistent with the involvement cryptochrome rather than other visual pigment[56]. Moreover, the iris photosensitivity was not affected by depletion of retinoid or inhibition of known visual transduction pathways[56]. Consistent with these results, knockdown of cryptochorme 1and 2, but not melanopsin decreased the light induced sphincter constriction[56].

Cryptochromes are a class of highly conserved, photoreceptive proteins, consisting of 50-70 kDa apo-proteins covalently bound to two flavin-cofactors[57]. Both cofactors are held within the N-terminal domain of the apo-protein. The C-terminal domain is of variable length and is involved in interacting with downstream signaling proteins[57]. Structurally, cryptochromes are similar to photolyases that catalyze the light-dependent repair of butane pyrimidine dimers[57]. Unlike photolyases, cryptochromes have lost the DNA repair activity, but have gained novel signaling functions. Cryptochrome proteins are present in both plants and animals. In plant, the photo-activation of cryptochrome leads to a conformational change, which in turn affects protein-protein interactions to regulate various developmental processes[58,59]. Based on their role in circadian rhythm, animal cryptochromes can be classified into two groups: type-I cryptochrome is light-responsive, directly feeding light information into the circadian clock; type-II cryptochrome, on the other hand, is thought to be light-irresponsive and to act as a transcription repressor[60–64]. Although classified as a type-II cryptochrome, chicken cryptochrome is able to act as a blue-light receptor responsible for the PMR of isolated chicken iris[56]. However, nothing is known about the downstream effectors that link the photo-activation of cryptochrome to muscle contraction.

The chicken iris sphincter muscle undergoes a smooth to striatal transition during development[55]. Interestingly, the PMR in chicken only exists for a short period (from embryonic day 12 to embryonic day 28), coincided with the time course of the disappearance of smooth muscle in the iris.

1.6 Objective

Despite the widely-accepted picture of the neutrally driven PLR, it has also been reported that even in isolation, the iris sphincter is capable of a light induced contraction. In mouse, the intrinsic pupillary light reflex (iPLR) in mouse requires melanopsin. In this study, we aim to understand the cellular and molecular mechanisms underlying the photosensitivity of mouse iris. To elucidate the mechanism of light response of the isolated mouse iris sphincter, we will identify the photoreceptive cell types in the mouse iris by genetic labeling method, and also examine the involvement of cholinergic synaptic transmission in activating light-induced muscle contraction. To identify the signaling components in the mouse iris, we will isolate the iris from knockout mouse lines missing one or more candidate genes, and examine the muscle's response to light and acetylcholine. Finally, given the recent concerns of overlabeling using very sensitive reporter lines, we will generate tissue-specific melanopsin mouse model, and investigate the effect of tissue-specific ablation of melanopsin expression on the PLR both *in vivo* and *in vitro*.

2. Experimental Procedures

2.1 Animals

The genetically-engineered mouse lines used in this study including: *Gαq*$^{f/f}$ (Ref 65), *Gα11*$^{-/-}$ (Ref 65), *Gα14*$^{-/-}$ (Ref 66), *Gα15*$^{-/-}$ (Ref 66), *Plcβ1*$^{-/-}$ (Ref 67), *Plcβ2*$^{-/-}$ (Ref 68), *Plcβ3*$^{-/-}$ (Ref 69), *Plcβ4*$^{-/-}$ (Ref 70) , *Itpr1*$^{-/-}$ (Ref 71), *Itpr2*$^{-/-}$*;Itpr3*$^{-/-}$ (Ref 72). *Gαq*$^{f/f}$*;Gα11*$^{-/-}$ double- and *Gαq*$^{f/f}$*;Gα11*$^{-/-}$*;Gα14*$^{-/-}$ triple-knockout lines were generated by crossing the corresponding lines. *Gαq*$^{f/f}$*;Gα11*$^{-/-}$ and *Gαq*$^{f/f}$*;Gα11*$^{-/-}$*;Gα14*$^{-/-}$ lines were crossed with *Opn4-Cre BAC* transgenic line (see below) to selectively knockout *Gαq*, $G\alpha_{11}$ and/or $G\alpha_{14}$ in melanopsin-expressing tissues. C57BL/6J (which was the genetic background for many of the lines) animals were used as WT controls. To obtain reproducible force measurement, the age of the animals use in this study was set as 3 months ± 5 days. For *Plcβ1*$^{-/-}$, 2-year-old animals were used due to limited number of homozygous animals. *Itpr1*$^{-/-}$ homozygous animals die before postnatal day 25-28, so 3-week old homozygous were used for experiments.

2.2 Generation of the *Opn4-Cre* line (By Wendy Yue, Laboratory of King-Wai Yau)

The Cre recombinase cDNA, followed by the rabbit β-globin poly-A signal, were inserted immediately after the start codon of exon 1 of the mouse *Opn4* gene in a bacterial artificial chromosome (BAC) clone (BACPAC Resource Center, RP23-340N18) by bacterial homologous recombination. Successful modifications were

confirmed by PCR and Southern blot. The modified BAC was linearized by enzyme digestion with AscI and SrfI, and subsequently injected into the pronuclei of B6SJLF2 embryos at the Transgenic Core Laboratory of Johns Hopkins University School of Medicine. Three transgenic founders were identified by PCR on genomic DNA (Forward primer: 5'- TGTGAA GGACAGAGCCTCCT -3'; Reverse primer: 5'- CAGCCCGGACCGACGATGAAG -3') and were bred with wildtype C57BL/6J mice to establish transgenic lines. One of these lines showed specific expression of tdTomato in ipRGCs when crossed to the Ai9 or Ai14 *Rosa-tdTomato* line–85% of melanopsin-immunopositive cells were tdTomato-labeled and 91% of cells showing tdTomato fluorescence were immunopositive for melanopsin (total 2342 cells from 3 animals analyzed). Some of the tdTomato$^+$, OPN4-immunonegative cells may be M4 or M5 ipRGCs, which were reportedly not stained by melanopsin antibody (AB-N38, Advanced Targeting Systems, 1:2500 dilution) under regular conditions (see below). Electrophysiologically, all cells labeled by *Opn4-Cre*-driven reporters (lines Ai9 and Ai14) and tested so far were intrinsically photosensitive (>150 cells). To quantify the number of reporter-labeled sphincter muscle cells in the iris, images of individual tdTomato-positive muscle cells was taken, and their lengths were measured by ImageJ. The number of melanopsin-expressing sphincter muscle cells per iris was calculated by dividing the total length of all groups of tdTomato-positive sphincter cells by the average length of individual cells (344 ± 58 μm, mean ± SD, n = 8).

2.3 Generation of the *floxed-opn4* line

Targeted C57BL/6N embryonic stem cells were obtained from UCDAVIS KOMP repository (clone EPD0642_3_D12), and subsequently injected into the B6(Cg)-*Tyr*$^{c-2J}$/J blastocysts at the Transgenic Core Laboratory of Johns Hopkins University School of Medicine. The mice generated from successful germline transmission were heterozygous for the knock-out first allele. Exposure of the construct to flippase [by *in vivo* breeding with ACTB:FLPe B6J mouse line (The Jackson Laboratory)] resulted in removal of the trapping cassette and expression of the *Opn4* gene. The second exon of *Opn4* was remain floxed, but was later removed conditionally after breeding to the *Myh11-Cre* mouse line (The Jackson Laboratory), resulting in a loss-of-function allele in smooth muscles. Genotyping primers for the floxed alleles are as follows: Floxed-opn4 (PostFlp) forward: 5'-TCTACACAGTGGCTGAGACAAGAGG-3', Floxed-opn4 (PostFlp) reverse: 5'-AAGAGGGAGTGAAAGGCTCAGATGG-3' (product 710bp); Wild type primers are the same as the primers for genotyping the floxed allele, except that the product is 554 bp.

2.4 Solutions

For iris-sphincter-muscle force measurement, the bath solution was bicarbonate-buffered Ames medium (Sigma). Cholinergic synaptic transmission was blocked by 10 μM Atropine (Sigma). 250 μM DL-2-Amino-4-phosphonobutyric acid (Sigma), 20 μM DNQX (Sigma), and 50 μM DL-2-Amino-5-phosphonopentanoic acid (Sigma) was used to block glutamatergic synaptic transmission. 1 μM

tetrodotoxin (Alomone Labs) was applied to muscle to block any action potentials from the residual ipRGC axonal terminals. 1 mM L-glutamic acid (Sigma), 100 nM PACAP (Pituitary Adenylate Cyclase-Activating Polypeptide) 1-27, 1-38 (Tocris, UK) was applied to the iris to test whether any contraction could be elicited. To reduce the desensitization effect of metabotropic glutamategic receptors, 100μM of cyclothiazide (Tocris) was applied together with glutamate. 100nM of PACAP 6-38 (Tocris) was used as antagonist for PACAP receptors. Two broad-spectrum gap junctional blockers octanol (500 μM, Sigma) and carbenoxolone (200 μM, sigma) were used for testing the role of gap junctions in the light-induced contraction of the isolated iris sphincter muscle.

2.5 Iris sphincter muscle force measurement

The isolated iris-sphincter-muscle was prepared as previously described [45]. An enucleated eye from an overnight dark-adapted mouse was used. The anterior chamber of the eye was initially excised by a circumferential cut along the ciliary body. The dilator muscle was subsequently trimmed away by using razor blade. Next, the iris sphincter muscle was separated from the cornea, transferred into the recording chamber superfused with Ames medium (equilibrated with 95%O_2/5%CO_2) at 36-37ºC and a flow rate of 3 ml/min. With infrared light and viewers, the muscle ring was mounted horizontally on an upright microscope between two stainless-steel hooks attached to micromanipulators. One hook was fixed and the other attached to a force sensor (see below). The muscle ring was slowly stretched to a length of 1.0-1.2 mm for 3-month old animals (for older

animals, the length of the muscle was 1.3-1.5 mm) which by trial and error was found to give roughly maximum force-generation.

Muscle force was measured with a fabricated device as previously published [45]. The device contains a single-crystal silicon strain-gauge with μ-Newton sensitivity (AE-801, Sensor One), and the signal was amplified with custom circuitry (Fig. 1). The force sensor was coated with a suspension of carbon powder in silicone in order to protect it from light and moisture. The voltage output of the sensor was proportional to the applied force (187 μN/V, calibrated by hanging various pre-measured weights fabricated from a thin silver wire), with a non-linearity of <0.1%. The signal was digitized by Digidata 1440A and acquired by pClamp 10.0.

For light responses, the muscle contraction was induced by delivering brief light flashes, where light intensity and duration are proportionally interchangeable without affecting the response. The light response of the mouse iris recovers quite slowly, so we separated light stimulation trials by≥10 min intervals. Hg-light was used in conjunction with an interference filter, centered at 436 nm (10 nm bandwidth). Light was delivered through a 5X objective as a uniform spot of 5-mm diameter on the muscle for mouse, large enough to cover the entire preparation. When white arc light was used on iris muscles for saturating the response, the white flashes were converted to equivalent 436-nm or 480-nm flashes by response-matching in the linear range with dim light.

The delivery of acetylcholine (ACh) and was controlled by solenoid valves. After a period of base line recoding, the bath-applied Ames medium was switched to

ACh of different concentrations. Once the steady state of a response was reached, the ACh was immediately switched off and the Ames channel was switched on to wash out the ACh.

2.6 *In situ* pupillometry

All animals were kept in 12 hr/12 hr light/dark cycle before experiments. The experiments were performed between 2 hours after lights-on and 2 hours before lights-off with >1-hr dark adaptation. To simultaneously monitor the pupillary light reflex in both eyes, we used a pupillometer with LED light for stimulation via a Ganzfeld sphere as previously descirbed[45]. The stimulated ipsilateral eye was monitored continuously with a miniature, infrared CCD camera (with an 850 nm long-pass filter) and illuminated with an infrared LED light (>850 nm) placed inside the Ganzfeld sphere, which have access to the sphere exterior via the opening of the Ganzfeld. The unstimulated contralateral eye was monitored with another infrared CCD camera with illumination from the infrared LED light. Light in the Ganzfeld interior was prevented from leaking out and stimulating the contralateral eye by using foam lining around the stimulated eye. Videos for both eyes were digitized and recorded at a frame rate of 5 Hz. A data-acquisition board (NI USB-6211, National Instruments) and custom software were used for triggering recordings and light stimulation simultaneously. The maximum pupil constriction in the ipsilateral, illuminated eye during the 30-sec light period was measured, and the pupil area of contralateral eye was measured at the same time.

2.7 Immunohistochemistry

All animals were anesthetized by intraperitoneal injection of ketamine (100 mg/kg body weight) + xylazine (5 mg/kg body weight) and perfused with phosphate buffered saline (PBS) followed by 4% paraformaldehyde in PBS. The eyes were enucleated, with all posterior tissue and lens removed. The irises and retinas were fixed for 30 min at room temperature in 4% paraformaldehyde in PBS, and blocked with 1% tyramide blocking reagent (Life Technologies) in 0.5% Triton X-100 in PBS over night at 4°C. The primary antibodies used in this study including polyclonal antibody against mouse melanopsin (AB-N38, Advanced Targeting Systems, 1:2500 dilution), polyclonal antibody against α-smooth muscle actin (ab21027, AbCam, 1:150 dilution), polyclonal antibody against M3 muscarinic receptor (AS3741S, Research and Diagnostics Antibodies, 1:250 dilution), polyclonal antibody against Pax6 (PRB-278P, Covance, 1:500 dilution), polyclonal antibody against GFP (A11122, Molecular Probes, 1:500 dilution), After washing, the tissue were exposed to secondary antibodies at 1:500 dilution for 3 hours at room temperature. Sections were mounted with anti-fade reagent containing 4',6'-diamidino-2-phenylindole (DAPI) and cover-slipped. Images were captured on a Zeiss LSM 510 confocal microscope.

For X-gal staining, Mouse irises and retinas were fixed in 0.2% glutaraldehyde in 0.1 M phosphate buffer containing 2 mM $MgCl_2$ and 5mM EGTA for 15 min. The tissue was then washed in 0.1 M phosphate buffer containing 2 mM $MgCl_2$, 0.01 % sodium desoxycholate and 0.02 % Nonidet P-40 for three times. Staining

was carried out at 37 °C (over night) in a solution of the above buffer containing X-Gal at a final concentration of 1 mg/ml, 5 mM $K_3Fe(CN)_6$, and 5 mM-$K_4Fe(CN)_6$.

For alkaline phosphatase staining, the tissues were fixed in 0.2% glutaraldehyde in 0.1 M Tris-HCl. The endogenous alkaline phosphatase activity was blocked by heat inactivation at 65 °C for 30min. Staining was carried by using Vector® Blue Substrate Kit (Vector labs) following manufacturer's instructions.

2.8 Quantitative RT-PCR

Total RNA was extracted from the mouse iris and retina using the TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. First-strand cDNA was synthesized from 2 µg DNase-treated total RNA using an oligo-dT15 primer and SuperScript® III reverse transcriptase (Invitrogen). Quantitative-PCR amplification and analysis were carried out with SYBR Green PCR Master Mix in an Applied Biosystems 7500 Real-Time PCR System by using the following primer sets: *Opn4S* (5'-GCTACCGCTCTACCCACC-3' and 5'-CTACATCCCGAGATCCAGACTG-3'), *Opn4L* (5'-GCTACCGCTCTACCCACC-3' and 5'-CACCTTGGGAGTCTTAGATCTCTG-3'), *β-actin* (5'-AAAGAGAAGCTG TGCTATGTTG-3' and 5'-CATAGAGGTCTTTACGGATGTC-3'). The specificity of the SYBR green PCR signal was further confirmed by a melting curve analysis and agarose gel electrophoresis. To estimate the relative abundance of two mouse *Opn4* isoforms, *Opn4S* and *Opn4L*, and standard DNA templates were generated by ligating the respective amplicon into the pGEM®-T Easy Vector (Promega). Standard curves were generated by plotting the threshold cycle (C_T)

against the log copy number of the starting standard DNA templates. The absolute copy number for *Opn4S* or *Opn4L* in each sample was calculated based on the standard curves, which were further divided by the absolute copy number of *β-actin* in the same sample. The abundance of individual isoform in iris or retina was normalized to that of *Opn4L* in the retina.

2.9 Knockdown of chicken *cryptochrome 1* and *2* (*cCry1 and 2*) by morpholino-modified oligonucleotides

Splicing-blocking morpholino oligos were chosen as gene knockdown reagent because its activity can be conveniently assayed by RT-PCR. Splicing-blocking oligos conjugated with 3'-fluorescein targeting the junction of the 2nd exon and the 2nd intron (E2I2) of *cCry1* (5'-TACGCATTTCTAGCATTTACCTTAA-3') and *cCry2* (5'-GAAAAGCAGTGTATTCTCTTACTTT-3') were synthesized, and electroporated (25V, 2 pulses/sec, 4 pulses on each side) into isolated irises from chicken embryo, followed by 24-48 hr culture post-electroporation. The knockdown efficiency was tested using the following primer sets: cCry1E1F: 5'-TGGTTCGCCGGCTCCTCCAA-3', cCry1E4R: 5'-CTGGCATCTCCAGTGGTTCCATCC-3'; cCry2E1F: 5'-CTCGTCGGCCGTGGGCATCA-3', cCry2E3R: 5'-CACCAGCCTCCTTGGCCAGTTT-3'. The wildtype bands for cCry1 and cCry2 are ~400 bp and ~200 bp, and the knockdown bands are ~300bp and ~100bp. Expression of β-actin was used as internal control (β-actin F: 5'-GACTGTTACCAACACCCACACC-3', β-actin R: 5'-CTTCACAGAGGCGAGTAACTTCC-3').

2.10 Cloning and expression of *cCry1* and *cCry2*

Total RNA was extracted from the irises of chicken embryo (embryonic day15, or E15) using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. First-strand cDNA was synthesized from 2 µg DNase-treated total RNA using an oligo-dT15 as primer and SuperScript™III Reverse Transcriptase as enzyme (Invitrogen). To obtain the full length chicken *Cry1* (*cCry1*) and *Cry2* (*cCry2*) sequences by PCR, the open reading frame of *cCry1* was amplified with Phusion High-Fidelity DNA Polymerase (NEB) using the gene specific primers (cCry1-5UTR: 5'- GCCGGGTCCTAGAGCCAT-3'; cCry1-3UTR: 5'-CCGGGTGTTAAGTGGTATCTCC-3'), and ligated into the pGEM®-T Easy Vector (Promega). The *cCry 2* open reading frame lacking the first 30 base pairs (*cCry2Δ30*) was amplified using the following primers: cCry2Δ30 F: 5'-TTTTGCCGCTCCGTGCAC-3', cCry2 R: 5'-TGAGCTCTTGCCAGGGATCTC-3', and ligated into the pGEM®-T Easy Vector (Promega). The 30 base pair nucleotide at the 5' end of *cCry2* [containing two silent nucleotide substitutions (underlined letters)] flanked by Apa1 and Sph1 restriction enzyme sites was synthesized (5'-CGGATCCCACCATGGCGGCGGCTGCGTCCCCGCCGCG<u>A</u>GG<u>A</u>GCATG 3'). The vector containing *cCry2Δ30* and the above sequence was digested with Apa1 and Sph1, and ligated with each other. Next, recombinant vectors containing full-length *cCry1* and *cCry2* were transformed into competent DH5α *E. coli*, and transformants were selected on LB plate containing 50 µg/ml ampicillin. Transformants were further analyzed for the presence of insert by restriction

digestion. Recombinant vectors from positive colonies were sequenced to confirm for correct insertion. The 4 base pair sequence (CACC) for directional cloning was included using the following primers: cCry1 topo F: 5'-CACCATGGGGGTGAACG-3', cCry1 topo R: 5'-ATTTGTGCTCTGCCGCTG-3'; cCry2 topo F: 5'-CACCATGGCGGCGGCTG-3', cCry2 R: 5'-TGAGCTCTTGCCAGGGATCTCCG-3'. Next, the above sequences were cloned into pENTR/D/TOPO Vector (Invitrogen). The recombinant vectors were transformed into competent TOP10 *E. coli*, and transformants were selected on LB plate containing 50 μg/ml kanamycin. Transformants were further analyzed for the presence of insert by restriction enzyme digestion. Recombinant vectors from positive colonies were sequenced to confirm the correct insertion. Mouse *cryptochrome 1* and *2* (*mCry1* and *2*) were amplified, and subcloned into pENTR/D/TOPO vector (mCry1F: 5'- CACCATGGGGGTGAACGC-3', mCry1R: 5'-GTTACTGCTCTGCCGCTGG-3'; mCry2F: 5'-CACCATGGCGGCGGCT-3', mCry2R: 5'- GGAGTCCTTGCTTGCTGGCTCT-3'). The inserts in the entry vectors were then cloned into the destination vector (pCIG-DV) using LR Clonase II Enzyme Mix (Invitrogen). The recombinant vectors were transformed into competent TOP10 *E. coli*, and transformants were selected on LB plate containing 50 μg/ml ampicillin. Recombinant vectors from positive colonies were sequenced to confirm for correct insertion.

Recombinant vectors containing *cCry1*, *cCry2*, *mCry1*, and *mCry2* were expressed in HEK293 cells. Protein extracts (15 μg) from each sample were separated by SDS-PAGE (12.5% polyacrylamide gel), and transferred to a

polyvinylidene fluoride (PVDF) membrane using BIO-RAD Mini-Trans-Blot SD Cell (transfer buffer: 0.25 M Tris, 0.2M Glycine, 20% Methanol). Anti-mouse CRY1 and CRY2 polyclonol antibody (from Developmental Studies Hybridoma Bank at University of Iowa) was diluted to 1:1000 in TBS-T (10 mM Tris-HCl, pH7.5; 150 mM NaCl, 0.1% Tween-20) containing 5% non-fat milk powder, and the secondary antibody of horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG was diluted to 1:2000 in the TBS-T containing 5% non-fat milk powder. Membranes were treated with SuperSignal West Pico Chemiluminescence Substrate (Thermo Scientific) for 1 min and exposed to x-ray film.

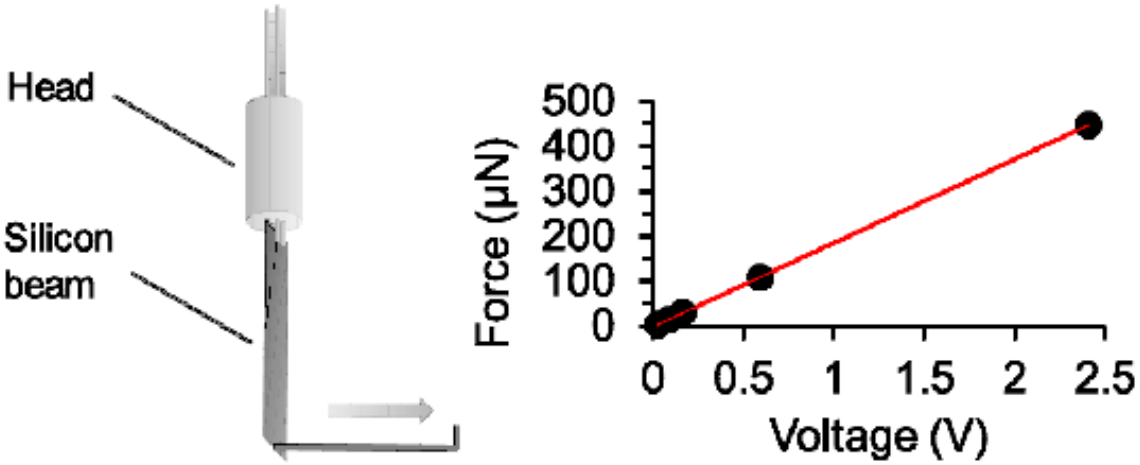

Figure 2-2 Force sensor and calibration. The force sensor element (left) consists of two parts, the silicon cantilever beam and the head that the beam is mounted. The bean is made of a single crystal N-type silicon and has one ion-implanted P type resistor on each side. The surface of the bean is passivated with thermally grown silicondioxide. The four pins of the head serve as both mechanical and electrical connections for the resistors. When the tip of the beam is deflected (to the direction indicated by the arrow), the resistor on the compressed side will decrease value, and the resistor on the other side will increase value. Consequently, the voltage output from each resistor will change. The plot on the right shows the relationship between change in voltage and force loaded onto the beam (1 V=187 μN).

3. Melanopsin signaling in mouse iris

3.1 ACh and light-induced contraction of isolated mouse iris sphincter muscle

The iris sphincter muscle was isolated from the eye, loop-mounted and stretched horizontally across two anchors with one anchor being connected to a micronewton (μN)-strain gauge for measuring isometric tension (Experimental Procedures). The muscle and anchors were submerged in a chamber continuously perfused with physiological saline solution at 36 ˚C.

Bath application of ACh produced a robust contraction of the isolated sphincter muscle, with little sign of adaptation at least over a period of seconds (Fig. 3-1A, also Ref 45). With an optimum stretching of the muscle across the anchors (Experimental Procedures), the dose-response relation gave a maximal isometric tensile force of 335.48 ± 52.11 μN (mean ± SD, 6 muscles), with a half-saturating ACh concentration ($K_{1/2}$) of 21.5 ± 11.4 μM (mean ± SD, 6 muscles).

A light flash also produced a muscle contraction (Fig. 3-1B and Ref 45). A light flash instead of a light-step was chosen for stimulation because the muscle did not readily recover from a light-step even at moderate intensity. The flash intensity-response relation gave a maximal isometric tensile force of 88.41±11.1 μN (mean ± SD, 6 muscles), with a half-saturating flash intensity (σ) of 1.97×10^{9} ± 6.45×10^{8} photons μm^{-2} (mean ± SD, 6 muscles), ~50-fold as high as that for mouse M1 ipRGCs[73]. In albino mouse, the iris sphincter muscle is more sensitive to light, due to the higher light penetration through the non-pigmented tissue (Fig.

3-4 C, legend). It is worthy of note that the maximum amplitude of muscle contraction, even to a step of light (Fig. 3-1B, top inset), was considerably smaller than the ACh-induced response. The large difference between the maximal muscle tensions elicited by ACh and by light described above may arise from an abundance of muscarinic receptors on the muscle, from the fewer number of melanopsin-expressing sphincter muscle fibers (see below).

The muscle's response to an intense flash often showed a hump during its decay (Fig. 3-1B), indicative of secondary contraction resulted from the initial response. Since the existence of gap junctions among the iris sphincter muscles has been reported based on anatomical studies[5], their functional contribution in the iPLR was examined by using pharmacological blockers. The initial peak to a light flash was largely preserved in the presence of 200 μM carbenoxolone (CBX). In contrast, the secondary hump was reversibly inhibited (Fig. 3-2A). The results were further confirmed by testing another gap junction blocker, 500 μM octanol (Fig. 3-2B). The data indicate that gap junctions mediate the propagation of responses originated from the photo-sensitive sphincter cells, leading to the active contraction of adjacent muscle cells.

When stimulated by light over a background of ACh, the sphincter muscles showed more complicated behaviors – the tension inexplicably decreased initially (Fig. 3-3) and then rose beyond the ACh-induced baseline tension. As the background concentration of ACh increased, the light-induced additional tension

became progressively smaller (Fig. 3-3), which may reflect the known influence of smooth muscle's passive tension on its active tension[74].

3.2 Light-Induced contraction of isolated sphincter muscle is independent of cholinergic transmission

We next asked whether ipRGCs' collateral axonal processes reported to be in the iris periphery[51–54] indeed, as suggested by others[51,52], underlie the local light response of the iris sphincter muscle (instead of our previous proposal of an intrinsic photosensitivity of the sphincter muscle) by presynaptically driving the parasympathetic cholinergic terminals innervating the muscle (see Introduction). After 1 hr bath-application of 1 μM tetrodotoxin (TTX) to block any potential light-induced spike activity in ipRGC axonal processes still on the isolated sphincter muscle albeit truncated, the muscle's flash response was unaffected (Fig. 3-4A). To exclude the possibility that melanopsin is expressed on ipRGC terminals and is able to drive the parasympathetic cholinergic terminals without involving action potentials, we blocked muscarinic ACh synaptic transmission from the parasympathetic innervation to the sphincter muscle by bath-applying 10 μM atropine (muscarinic receptor antagonist). This treatment completely removed the muscle's response to ACh, but again did not affect its response to light (Fig. 3-4B, top and bottom). Finally, in irises from mice with genetically-ablated M_1 and M_3 muscarinic receptors [i.e., *Chrm1*$^{-/-}$;*Chrm3*$^{-/-}$ mice; at least M_1 and M_3 muscarinic receptors are known to be on the iris sphincter muscle[17,20,21]], the muscle's response to ACh disappeared but its response to a bright flash remained essentially the same as WT (Fig. 3-4C). The *Chrm1*$^{-/-}$,*Chrm3*$^{-/-}$ mice we

obtained so happened to have an albino genetic background, explaining their iris sphincter muscle being more photosensitive due to a higher light penetration through non-pigmented tissue (see Fig 3-4C legend). Together, these findings ruled out the possibility of ipRGCs being the activator of the sphincter muscle contraction indirectly via the muscarinic cholinergic synaptic transmission as proposed by others[51,52].

IpRGCs are known to release glutamate and PACAP[75,76]. Vertebrate muscle is not known to have glutamate receptors, but for thoroughness we decided to examine this remote possibility. Bath-application of a mixture of inhibitors for ionotropic and metabotropic glutamate receptors (20 μM DNQX for AMPA and kainite receptors, 50 μM DL-2-amino-5-phosphonopentanoic acid for NMDA receptors, and 250 μM DL-2-amino-4-phosphonobutyric acid for metabotropic glutamate receptors) hardly reduced the light response (Fig. 3-5A, top). 1 mM glutamate with or without 100 μM cyclothiazide (for reducing the desensitization of AMPA receptors) also did not elicit any muscle contraction (Fig. 3-5A, middle and bottom). Finally, 100-nM PACAP-receptor agonists (PACAP 1-27 and 1-38) did not elicit any muscle contraction, nor did 100-nM PACAP-receptor antagonist (PACAP 6-38) reduce the light response (Fig. 3-5B). The PACAP experiments are nonetheless only suggestive because PACAP agonists and antagonist, being peptides, might not have full access to the postsynaptic sites on the muscle. Despite this uncertainty, it remains a more likely scenario that some iris sphincter muscle cells are intrinsically photosensitive, as we suggested previously[45] and substantiated with genetic-labeling below.

3.3 Melanopsin-expression in mouse iris

Given the above physiological findings, we sought to visualize melanopsin expression in the mouse iris sphincter muscle. We first confirmed our previous finding that melanopsin transcripts are present in the mouse iris. By quantitative RT-PCR (Experimental Procedures), both splice variants of melanopsin, *Opn4S* and *Opn4L*, were detected in the iris, with *Opn4S* expressed at a level ~2 times that of *Opn4L* (Fig. 3-6). Interestingly, there is actually higher melanopsin message in the iris than in the retina, but the higher expression of *Opn4S* than *Opn4L* holds true in both. The functional implication of this differential abundance in isoforms is currently unclear (See also Ref 77,78). Next, we checked by immunohistochemistry melanopsin protein expression on whole-mount mouse irises. Despite a mildly-higher overall immunosignal found in the sphincter-muscle region, no distinct muscle cells or axon-like structures could be unequivocally labeled, broadly similar to our earlier immunohistochemical results on cryosections of the mouse iris[45]. The ambiguous melanopsin-immunosignal in the mouse iris was possibly due to its low protein expression level, as, for example, in M4 and M5 ipRGCs[79].

We turned to genetic labeling. With X-gal labeling of the iris from albino *Opn4*$^{\tau lacZ/+}$ mice[80] we found no clear signal in the iris despite good labeling in the retina (Fig. 3-7). Previously, with *Opn4-tdTomato* BAC transgenic mice, we did detect tdTomato fluorescence, albeit dim, in the iridic region next to the pupil, which is where the sphincter muscle is located[45]. To enhance the melanopsin signal, we generated an *Opn4-Cre* BAC transgenic mouse line and crossed it to

a *Rosa-tdTomato* (Ai9) or a *Rosa-Alkaline Phosphatase* (R26iAP) reporter line so as to exploit the signal amplification provided by the *Rosa26* promoter and possibly also by the alkaline-phosphatase-mediated chromogenic reaction. Specificity of the Cre-mediated recombination was confirmed in the retina by the correct labeling of ipRGCs (Fig. 3-8 and legend). Importantly, as with other published *Opn4-Cre* lines[79,81], our *Opn4-Cre* line was able to reveal even retinal ipRGCs (such as M4 cells) that had no obvious melanopsin-immunohistochemical signal but had been functionally confirmed to be indeed intrinsically-photosensitive with electrophysiology.

In the iris from albino *Opn4-Cre;R26iAP* mice, we found ~30 labeled sphincter muscle cells per iris with characteristic location and shape (Fig. 3-9A; Experimental Procedures). These labeled cells represented only a small percentage (~10%) of all sphincter muscle cells (total number of sphincter muscle cells see Ref 82). Similar labeling was observed in the *Opn4-Cre;Ai9* iris (Fig. 3-9B), with *Opn4-Cre* labeled cells co-expressing α-smooth muscle actin (αSMA), a smooth-muscle marker (Fig. 3-10A), and M3 muscarinic receptor (Fig. 3-11C). At least the great majority of sphincter muscle cells, if not all, appeared to have the M3 muscarinic receptor (Fig. 3-11B). As such, it is likely, although not absolute, that every melanopsin-expressing sphincter muscle cell also has M3 receptors. The fact that a saturating (1 mM) concentration of background ACh occludes the light-induced muscle tension (Fig. 3-3) is not inconsistent with this notion.

Interestingly, based on *Opn4-Cre;R26iAP* and *Opn4-Cre;Ai9* irises, melanopsin seemed to be expressed also in the dilator-muscle region (Fig. 3-9), not in the muscle cells but in cells located to the posterior layers of the iris (Fig. 3-11A and legend). The colocalization of the melanopsin signal with PAX6 (Fig. *Opn4$^{f/f}$*, the conditional allele B), a transcription factor present in essentially all adult iris smooth muscle cells and also iris pigmented epithelial (IPE) cells[83,84], suggests that these are possibly IPE cells. Occasionally, seemingly the same cells were found in the sphincter-muscle region (Fig. 3-9). It is at present not clear whether such labeling in the dilator-muscle region represents real melanopsin protein expression (versus, for example, transient activation of the melanopsin promoter during development) and if so, whether there is any functional implication.

Consistent with an earlier report[54], we also observed a plexus of melanopsin-immunolabeled processes at the edge of the mouse retina (Fig. 3-12). However, we did not detect any obvious invasion of these processes into the ciliary body or iris as reported by Ref. 54.

3.4 Tissue-specific ablation of melanopsin-expression in smooth muscle

Given the recent report about the concern of overlabeling using very sensitive reporter line[85], we decided to demonstrate the presence of melanopsin in the iris smooth muscle by reverse genetics. To achieve this goal, the targeted embryonic stem (ES) cells (see Attachment 1) was purchased from UC Davis KOMP, and produced a knockout-first animal. The expression of *Opn4* in those animals were disrupted due to the large insertion inside the first intron of *Opn4* (Fig. 3-13A). By

crossing with a germ-line expressed flippase line, the non-expressive allele (i.e. *Opn4*$^{KOF/KOF}$) was then converted to the conditional allele (i.e. *Opn4*$^{f/f}$, Fig. 3-13A). The floxed melanopsin line was then crossed with a smooth muscle-Cre line. The *smMHC/Cre/eGFP* transgene was designed by placing a *Cre* recombinase gene, internal ribosomal entry site (*IRES*), and an enhanced green fluorescent protein (*EGFP*) gene all downstream of the 16 kb mouse smooth muscle myosin heavy chain (*smMHC* or *Myh11*) promoter fragment. The expression of Cre in the iris sphincter muscle was examined by staining using the anti GFP antibody (Fig. 3-13B). There is good percentage of sphincter muscle cells express GFP. The expression of Cre should be even higher because the GFP was placed downstream of the *IRES* element. The light response of the iris isolated from this conditional knockout lines will be examined after obtaining the mice with desired genotype.

The conditional melanopsin knockout also allowed us to probe the functional contribution of iPLR *in vivo*. Because of the iPLR, the overall pupil constriction in an illuminated eye should be stronger than the consensual constriction in the contralateral, unilluminated eye. Accordingly, we have simultaneously monitored both pupils of a mouse with infrared CCD camera, while subjecting one eye to light stimulation. The difference in pupil area of the ipsilateral (illuminated) and contralateral (unilluminated) eye is most evident under medium light intensity (Fig.3-14). Under higher light intensity, the ipsilateral illuminated pupil is still smaller than the pupil of the contralateral eye (Fig.3-14). We will do the same

experiment using the conditional melanopsin knockout animals, and provide a quantitative analysis of the contribution of the iPLR *in vivo*.

3.5 Melanopsin signaling mechanism in sphincter muscle

Previously, we have found that the phototransduction mechanism underlying the intrinsic pupillary light reflex involves a phospholipase C (PLC)-signaling pathway as in ipRGCs[45]. Given that melanopsin indeed is expressed in the mouse iris sphincter muscle, we decided to examine its signaling in more detail, together with muscarinic signaling. The isolated sphincter muscle was again used in these experiments.

We first addressed the identity of the mediating G-protein. Given that PLC is the enzyme underlying the sphincter muscle contraction, we examined the four members of the $G_{q/11}$-subfamily. Because unconditional $G\alpha_q^{-/-}$ mice have high embryonic lethality, we studied instead the *Opn4-Cre;*$G\alpha_q^{f/f}$ genotype, in which $G\alpha_q$ was knocked out only in melanopsin-expressing cells. The flash-induced tension of *Opn4-Cre;*$G\alpha_q^{f/f}$ sphincter-muscle was not attenuated (Fig. 3-15A). Nonetheless, when $G\alpha_{11}$ was also knocked out (i.e., with *Opn4-Cre;*$G\alpha_q^{f/f}$;$G\alpha_{11}^{-/-}$ genotype), the flash-induced tension decreased by up to 80% (Fig. 3-15A) at the highest intensity tested, suggesting functional redundancy between $G\alpha_q$ and $G\alpha_{11}$. This large effect was in contrast to the situation in ipRGCs, in which the *Opn4-Cre;*$G\alpha_q^{f/f}$;$G\alpha_{11}^{-/-}$ genotype only mildly reduced the light response[59]. Knocking out $G\alpha_{14}$ additionally (i.e., with *Opn4-Cre;*$G\alpha_q^{f/f}$;$G\alpha_{11}^{-/-}$;$G\alpha_{14}^{-/-}$ genotype) did not further reduce the light-induced muscle response (Fig. 3-15A), indicating that

Gα$_{14}$ has a negligible role. The still-incomplete removal of the *Opn4-Cre;Gα$_q^{f/f}$;Gα$_{11}^{-/-}$;Gα$_{14}^{-/-}$* muscle's light response might arise from *Opn4*'s promoter being not strong enough to drive *Cre* expression and ablate *Gα$_q$* in all melanopsin-positive cells. The reduction in muscle's light response associated with both the *Opn4-Cre;Gα$_q^{f/f}$;Gα$_{11}^{-/-}$* and the *Opn4-Cre;Gα$_q^{f/f}$;Gα$_{11}^{-/-}$;Gα$_{14}^{-/-}$* genotypes were not from a defective contractile apparatus because the muscle's response to ACh was only moderately reduced (Fig. 3-15B). This weak effect on the overall ACh response is not surprising because the mutations only ablate *Gα$_q$* in melanopsin-expressing muscle cells but not the great majority of muscle cells, which also bear muscarinic receptors (see above). Because the *Gα$_{15}$* and *Gα$_{11}$* genes are tightly linked, we were not able to produce quadruple-KO mice, but *Gα$_{15}^{-/-}$* single-KO muscles showed practically no change in light response from WT (Fig. 3-15A).

Regarding PLC, we previously showed that the *Plcβ4$^{-/-}$* genotype reduced the sphincter muscle's light response to ~10% of wild type (WT)[45]. We checked here the possible involvement of the other three PLC isoforms, PLCβ1-3. *Plcβ1$^{-/-}$* and *Plcβ3$^{-/-}$* single-KO sphincter muscles showed little deficit (Fig. 3-16A). In contrast, *Plcβ2$^{-/-}$* muscles showed severe defect in their response to a light flash (Fig. 3-16A), essentially the same as observed from *Plcβ4$^{-/-}$* muscles[45]. That both *Plcβ2$^{-/-}$* and *Plcβ4$^{-/-}$* single-KO muscles showed greatly diminished light responses was not due to a reduced expression of *Plcβ4* in iris *Plcβ2$^{-/-}$* or vice versa. One possible explanation is that a critical level of PLC enzymes is required for supporting downstream signaling. We were unable to produce the *Plcβ2$^{-/-}$;Plcβ4$^{-/-}$*

double-KO genotype because the *Plcβ4*$^{-/-}$ single-KO animals were weak, making their cross-progenies difficult to generate. Interestingly, the responses of *Plcβ2*$^{-/-}$, *Plcβ3*$^{-/-}$ and *Plcβ4*$^{-/-}$ single-KO sphincter muscles to ACh were all only slightly smaller than in the WT situation (Fig. 3-16B). The simplest explanation is that the light-responsive sphincter muscle cells express a more restrictive set of PLC isoforms (PLCβ2 and PLCβ4) than do light-unresponsive cells, thus ACh signaling enjoys a higher PLC-isoform redundancy. Alternatively, melanopsin and ACh signaling may be segregated at subcellular level (e.g. in lipid rafts, see Ref 43) so that they exclusively recruit certain PLC isoforms.

In smooth muscle, the inositol-trisphosphate receptor (IP_3R) interposes between the second messenger, IP_3, produced by PLC and the intracellular release of Ca^{2+}, which triggers muscle contraction. Of the three known IP_3R isoforms (IP3R1-3), IP3R1 is the predominant isoform expressed in smooth muscles[71,86]. Indeed, the *Itpr1*$^{-/-}$ iris sphincter muscle has lost its response to both light and ACh (Fig. 3-17A). On the other hand, *Itpr2*$^{-/-}$*;Itpr3*$^{-/-}$ double-KO iris sphincter muscles responded essentially normally to a light flash (Fig. 3-17B). Thus, IP3R1 may be the point of convergence for the ACh- and light-signaling pathways.

3.6 Discussion

Based on experimental results presented above, we arrived at an overall picture about how light triggers contraction of the iris sphincter muscles – light directly activates a $G\alpha_q$/$G\alpha_{11}$-PLCβ2/ PLCβ4-IP_3R1-mediated phototransduction pathway in a small subpopulation of sphincter muscle cells distinguished by their

expression of the visual pigment, melanopsin, leading to an initial contraction. The activation in this cohort of cells propagates to neighboring conventional muscle cells via gap junctions, producing a secondary contraction that is manifested as a delayed hump in the muscles' light response. Contractions of the muscle elicited by ACh seem to go through additional pathways but converge with phototransduction at the step of actin-myosin interaction triggered by a rise in intracellular Ca^{2+} concentration.

There is no evidence to support the proposal by others[51–54] that the light signal triggering the sphincter muscle's contraction goes sequentially through the intraocular ipRGC axonal collaterals, the parasympathetic presynaptic terminals, and finally to the muscle via muscarinic synaptic transmission. First, light-induced contraction was preserved and lasted for hours even in the reduced preparation of isolated sphincter muscles, in which ipRGCs' collateral processes, if any, would have been greatly severed. Second, the sphincter muscles' light response remained intact in atropine, even though the ACh response was blocked. Finally, we have not observed any melanopsin-positive neuronal innervations in the sphincter muscle of mouse with multiple histochemical and genetic-labeling methods. We did, however, detect convincing labeling of sphincter muscle cells (defined by αSMA-expression) in the *Opn4-Cre;R26iAP* and *Opn4-Cre;Ai9* irises. IpRGCs' axon collaterals populating near the ciliary body have been suggested to facilitate iPLR *in vivo*[53,54]. Given that the pupil size is antagonistically regulated by both the iris sphincter and dilator muscles, it remains an interesting question as to whether these ipRGCs' axonal processes exert their control over the dilator

muscle, instead of the sphincter muscle, for example, by innervating the dilator muscle directly, or indirectly via the autonomic fibers.

The *in vivo* action of atropine is complex, which could block iPLR in some study[54] but not the other[87]. Base on the histological and functional studies on the double reciprocal innervation by cholinergic and adrenergic nerve fibers to both iris muscles[1,8], application of atropine would lead to opposite effects on the sphincter versus the dilator muscle, which blocks the action of ACh on the sphincter muscle thus abolishing pupil constriction, but activates the dilator muscle due to the removal of presynaptic inhibition of NE release[26,27]. The length of individual sphincter muscle cells is 40% longer in a fully dilated state compared with that in the 50% constricted state. Based on the known length-tension relation of smooth muscle cells, the active tension (agonist-elicited tension) is expected to reduce to zero when the length of the muscle cell exceeds 120% of baseline length[74]. Duration of mydriasis after administration of 1% atropine sulfate in the mouse eye is greater than 8 days, which has been attributed to binding of the drug to melanin pigments found in iris tissue and slow release of accumulated drug onto receptors[88,89]. Consequently, the sphincter muscle would be trapped in a passive state for prolonged period, thus no activation could be produced by neither ACh nor light. In the *in vitro* prep, the action of dilator muscle has been eliminated, leaving only the sphincter muscle being functional.

The finding that a small percentage of iris sphincter muscle cells contain the visual pigment melanopsin is interesting on two counts. First, a concatenation of

classical sensory process (the reception of light) and effector process (the production of force) could happen inside a single smooth muscle cell. Second, it encourages studies on the development of these photoreceptive sphincter muscle cells since the mammalian iris sphincter muscle is a very rare case of ectodermally, rather than mesodermally derived muscle[83], and has long been thought to be derived from the same stem cells as are the photoreceptors[4]. In view of the findings in the present study, it would be intriguing to examine the origin of mammalian iris photoreceptive sphincter muscle cells by tracing the fate of the genetically labeled cells.

The melanopsin-expressing muscle cells did not tile the entire sphincter muscle area. In contrast, they located in areas that are closer to the edge of the iris. It is worthy of note that the location of the labeled muscle cells are not in perfect symmetry, which raises concerns about the consequence of the light-activated muscle contraction. Nonetheless, the presence of gap junctions on the muscle cells overcomes the limitation caused by the anatomical location of the light-responsive muscle cells, thus producing a uniform pupil constriction.

There is a growing evidence suggesting that melanopsin signals via the $G_{q/11}$ type of G-proteins. However, the precise identity of the Gα subunits involved in melanopsin phototransduction remains to be determined by using knockout lines lacking one or more of the candidate genes. In retina, Hughes and colleagues[90] reported the localization of $G\alpha_q$, $G\alpha_{11}$ and $G\alpha_{14}$ in mouse iPRGCs. Furthermore, by combining *in vitro* and *in vivo* siRNA, they demonstrated that melanopsin are

capable of coupling with $G\alpha_q$, $G\alpha_{11}$ and $G\alpha_{14}$ both in heterologous system and *in vivo*. In iris, our study provides the first genetic analysis of melanopsin and G-protein interactions. Using irises isolated from individual knockout mouse lines, we demonstrate an overlapping role for $G\alpha_q$ and $G\alpha_{11}$ in melanopsin driven intrinsic pupillary light responses (iPLR). Light-induced contraction of the isolated mouse iris sphincter muscle was largely unaltered in mice lacking *Gα$_q$* specifically in melanopsin-expressing muscle cells, whereas simultaneous silencing of *Gα$_q$* and *Gα$_{11}$* subunits resulted in a significant attenuation of iPLR in a manner comparable to that has been observed following removal of *Plcβ4*[45]. Given that knocking out *Gα$_{14}$* or *Gα$_{15}$* alone did not result in a deficit in muscle's light responses, we can conclude that melanopsin is capable of coupling to both $G\alpha_q$ and $G\alpha_{11}$ *in vivo*.

The efficiencies with which $G\alpha_q$ and $G\alpha_{11}$ activate PLCβ isoforms are equally well both in cells and after purification[91]. Both subunits are potent activators of PLCβ1, β3 and β4. PLCβ2 is the least sensitive to Gαq but is still activated more than ten folds[91]. PLCβ2, β3, and β1 are also activated by the Gβγ subunits, and PLCβ2 is the most Gβγ sensitive isoform. Given the significant reduction in the light-activated muscle contraction after removing either PLCβ2 or PLCβ4, the simplest explanation is that the two PLCβ isoforms co-exist in the melanopsin-expressing sphincter muscle cell, and function as dimmers.

Melanopsin expression in sphincter muscle cells is the first established case of a functional presence of an opsin in mammalian non-neuronal tissues. Multiple

opsins, including rhodopsin, have been suggested to be expressed in human epidermal cells but their functions are not clearly delineated[92].Melanopsin has been reported to mediate relaxation of the blood vessels in mouse[93]. We have also recently found a circadian function of another opsin, OPN5, in the mouse cornea[94]. However, in both cases, the identities of opsin-expressing cells are still unclear.

In conclusion, our findings offers a molecular and genetic analysis of the iris-based photoreceptive sphincter muscle cells in mouse. Our data demonstrate the ability of these muscle cells to be excited directly by light, as well as the mechanism of intracellular control of the melanopsin-mediated photoreception, in contrast to that in the ipRGCs, involving a distinct set of G-proteins and downstream effectors.

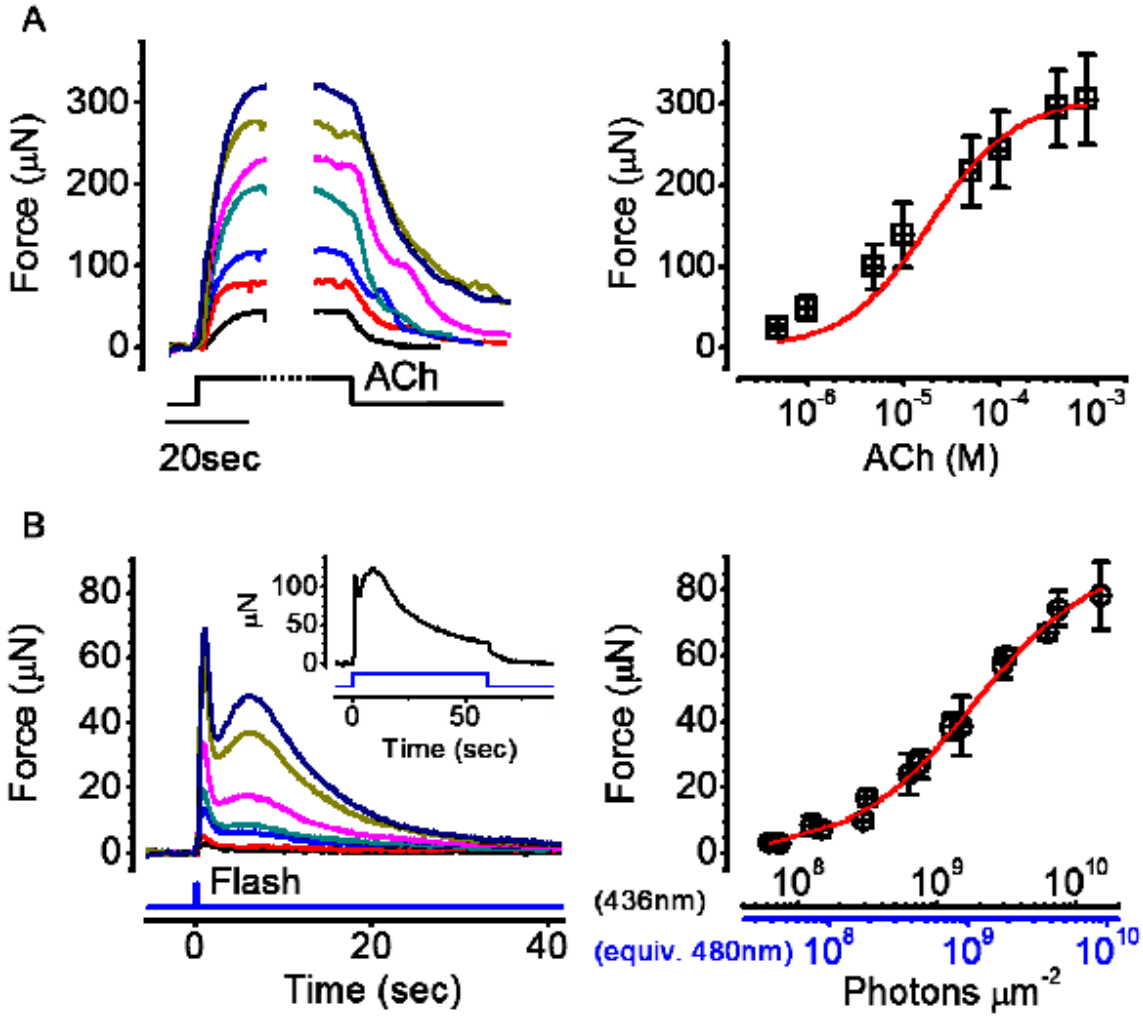

Figure 3-1 Segregation of ACh- and light-signaling pathways in iris sphincter muscle. A, left panel, muscle force response to steps of bath-applied ACh (1, 5, 10, 50, 100, 400, and 800 μM). Each trace is aligned at the on- and off-set of the stimulation. Right panel, dose-response relation of isolated iris sphincter muscle to steps of ACh (mean ± SD., 6 muscles). Fit is Hill equation

(red line). B, left panel, muscle-force response to light flashes (6.27×10^{7}, 1.25×10^{8}, 3.14×10^{8}, 6.27×10^{8}, 1.25×10^{9}, and 3.14×10^{9} photons μm^{-2}, at 436 nm). The inset shows a sample response to a step of light (3.077×10^{9} photons μm^{-2} sec^{-1}, 60sec step at 436 nm). Right panel, flash-intensity-response relation for sphincter muscle force at the transient peak of the response (mean ± SD., 6 muscles). Flash intensities are also expressed in equivalent 480 nm photons, given that melanopsin is the signaling pigment[45]. Fit is Michaelis-Menten equation (red line).

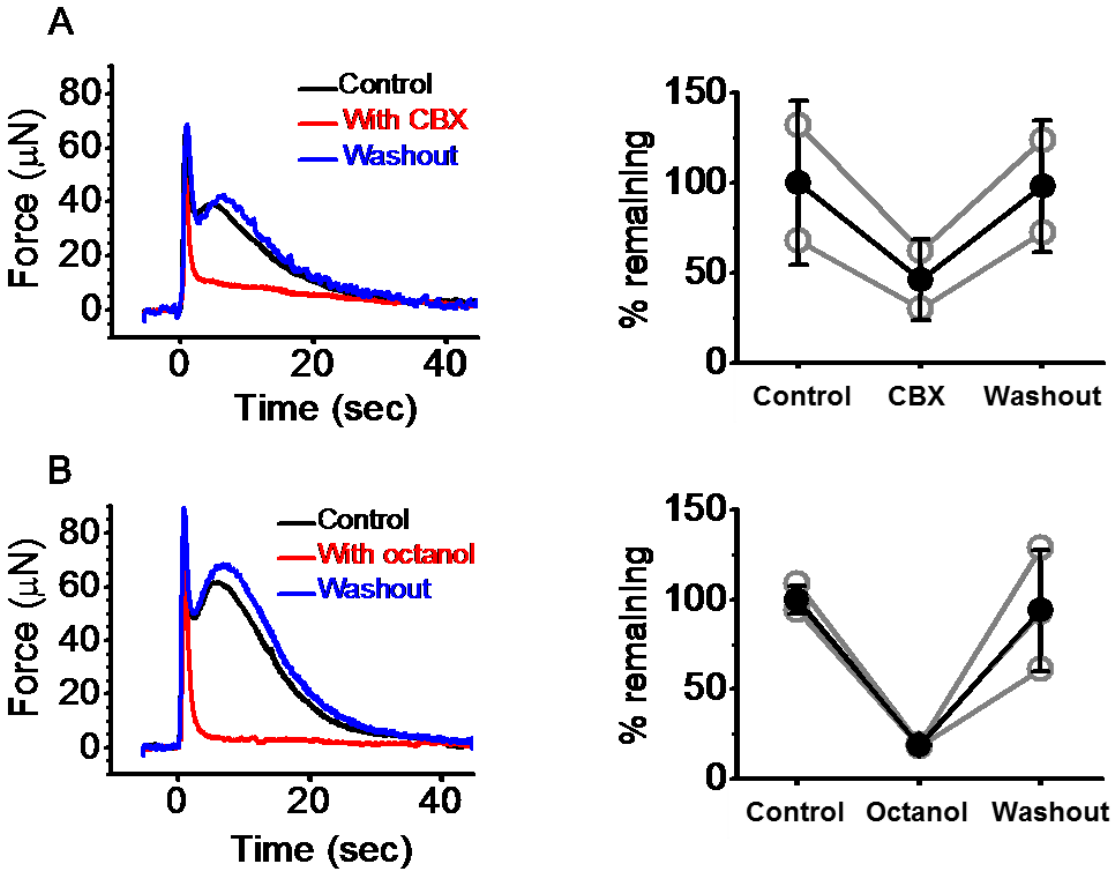

Figure 3-2 Involvement of gap junctional coupling in light-induced contraction of isolated iris sphincter. A, left, sample responses to a light flash (7.38×10^9 photons μm^{-2} at 436 nm) in control solution (black), with 200 μM CBX (red), and after washout (blue). Right, collective data of integrated response amplitude to light flash with and without CBX response (mean ± SD., 3 muscles). B, left, sample responses to a light flash (7.38×10^9 photons μm^{-2} at 436 nm) in control solution (black), with 500 μM octanol (red), and after washout (blue).

Right, collective data of integrated response amplitude to light flash with and without octanol response (mean ± SD., 2 muscles).

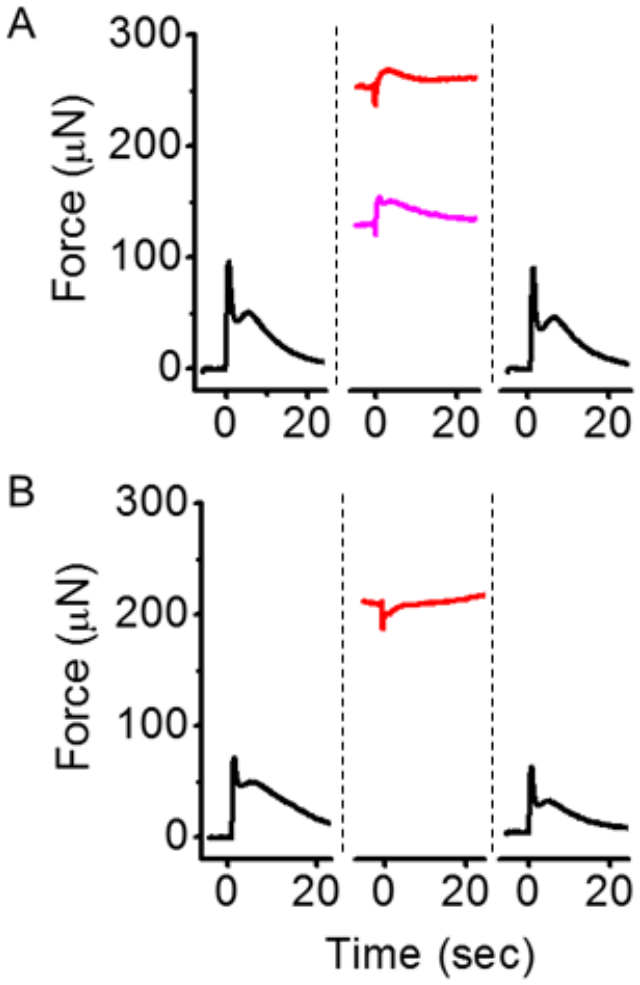

Figure 3-3 Light responses of isolated iris sphincter muscle with or without ACh exposure. A, muscle contraction to a light flash (left panel, 7.38×10^{9} photons μm^{-2} at 436 nm) was diminished when co-applying a step of 100 μM ACh (middle panel, magenta trace) or 400 μM ACh (middle panel, red trace). After withdrawal of bath-applied ACh, the response to the light flash of the same intensity returned back to normal (right panel). B, muscle contraction to a light flash (left panel, 7.38×10^{9} photons μm^{-2} at 436 nm) was completely inhibited

when co-applying a step of 1 mM ACh (middle panel). After withdrawal of bath-applied ACh, the response to a light flash of the same intensity partially recovered (right panel).

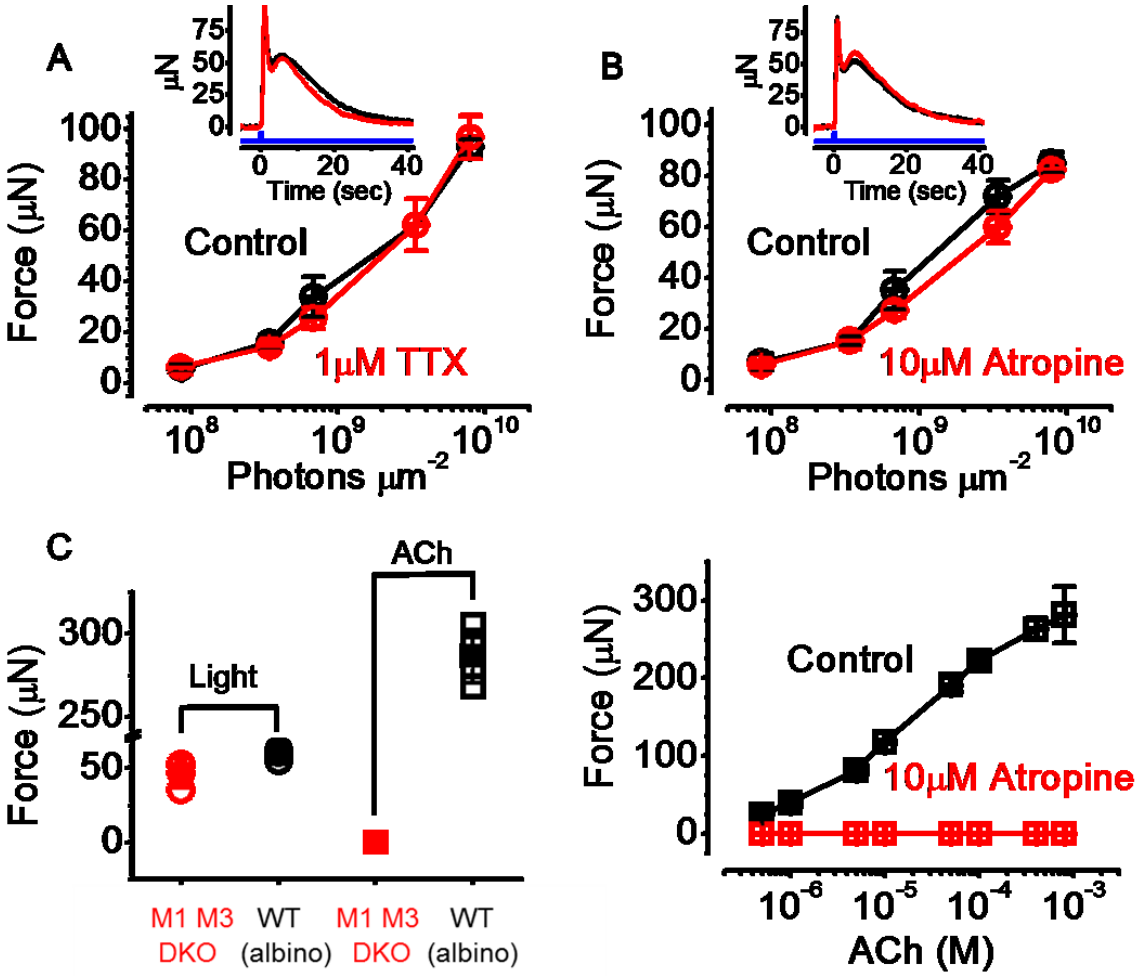

Figure 3-4 Effect of TTX and atropine on iPLR. A, intensity-response relations of isolated WT sphincter muscle to light flashes with (red) and without (black) TTX (mean ± SD, 3 muscles). The inset shows sample response of the isolated WT iris sphincter muscle to a light flash (7.95×10^{9} photons μm^{-2} at 436 nm) before (black) and in the presence of 1 μM TTX (red). B, upper panel, intensity-response relations of isolated WT sphincter muscles to light flashes with (red)

and without (black) atropine (mean ± SD, 3 muscles). The inset showed sample response of the isolated WT iris sphincter muscle to a light flash (7.95×10^{9} photons μm^{-2} at 436 nm) before (black) and in the presence 10 μM atropine (red). Bottom panel, 10 μM atropine completely blocked muscle's response to ACh (black: control; red: with 10 μM atropine; mean ± SD, 3 muscles). C, response amplitude of irises isolated from *Chrm1*$^{-/-}$*Chrm3*$^{-/-}$ (red) vs. WT-albino (black) animals to light flash (4.90×10^{7} photons μm^{-2} at 436 nm) and ACh (1mM).

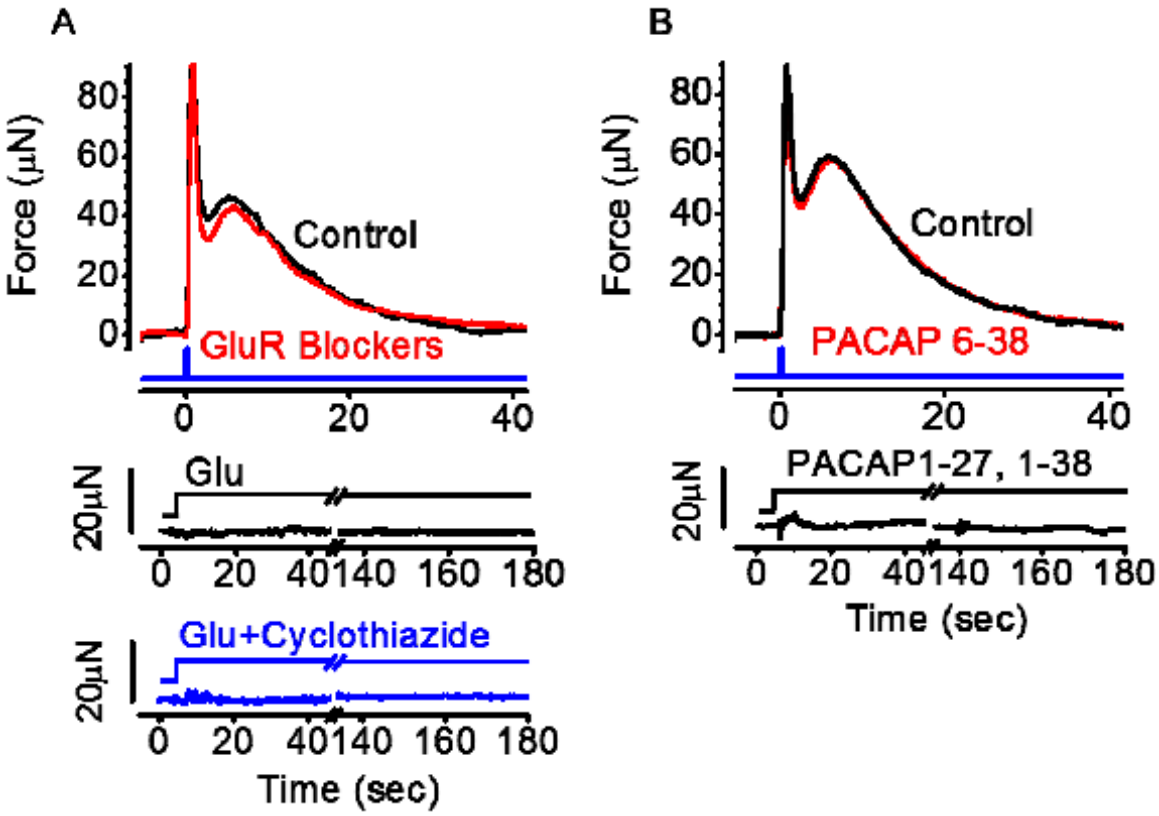

Figure 3-5 Effect of Glutamate and PACAP receptor blocker on iPLR. A, sample response of isolated WT iris sphincter muscle to a light flash (7.95×10^{9} photons μm^{-2} at 436 nm) in control solution (black) vs. in bath solution containing 20 μM DNQX, 250 μM DL-2-Amino-4-phosphonobutyric acid, 50 μM DL-2-Amino-5-phosphonopentanoic acid (red). Middle and bottom panels, 1 mM glutamate or 1 mM glutamate with 100 μM cyclothiazide did not induce any muscle contraction. B, upper panel, sample response of isolated WT iris sphincter muscle to a light flash (7.95×10^{9} photons μm^{-2} at 436 nm) in control solution (black) vs. in solution containing100-nM PACAP 6-38. Lower panel, 100

nM PACAP 1-27 and 1-38 did not induce any contraction on the iris sphincter muscle.

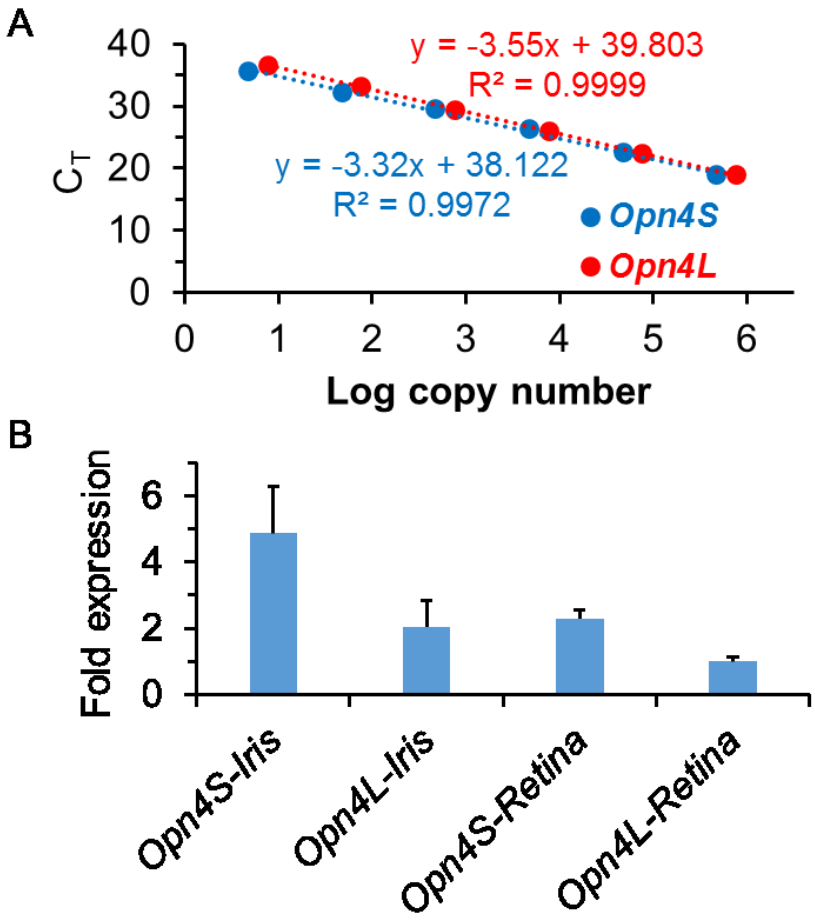

Figure 3-6 Expression of *Opn4S* and *Opn4L* in the mouse iris and retina. A, standard curves were generated using duplicate ten-fold serial dilutions of *Opn4S* and *Opn4L* standard DNA templates (see Experimental Procedures), plotted by threshold cycle (C_T) vs. log copy number of the starting template. B,

relative expression of *Opn4S* and *Opn4L* in mouse iris and retina (mean ± SD, n=3; corrected for amplification efficiency, see Experimental Procedures).

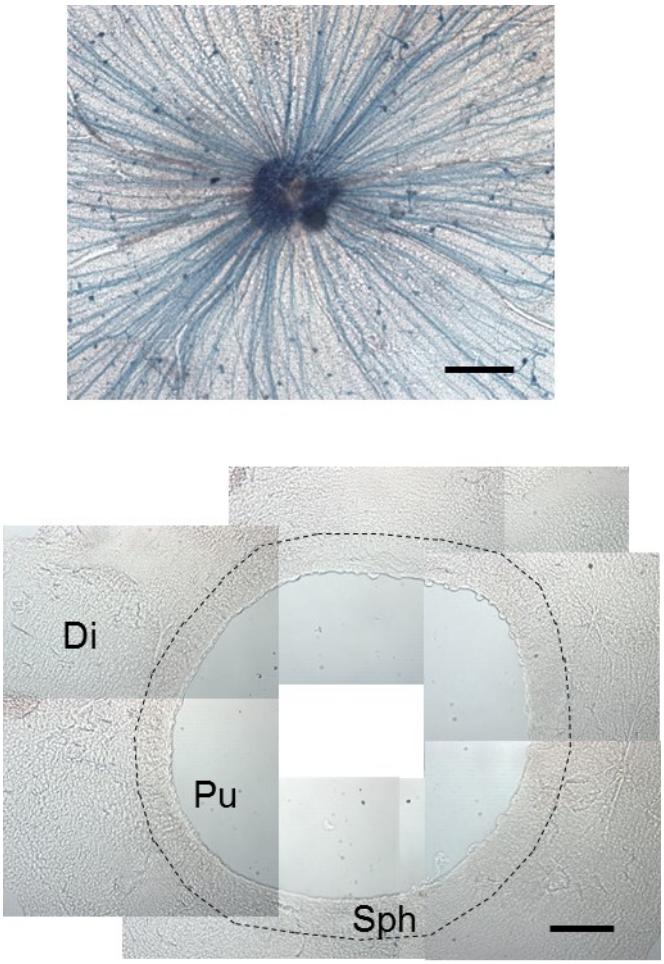

Figure 3-7 X-gal staining of iris and retina isolated from the *Opn4*$^{\tau lacZ/+}$ line. Upper panel, whole-mount mouse retina stained with X-gal. Both labeled axons and cell bodies are visible. Lower panel, whole-mount mouse iris staining with X-gal. No clear signal could be identified in neither sphincter (Sph), nor dilator (Di) region. Pu: pupil. Scale bar = 200 μm.

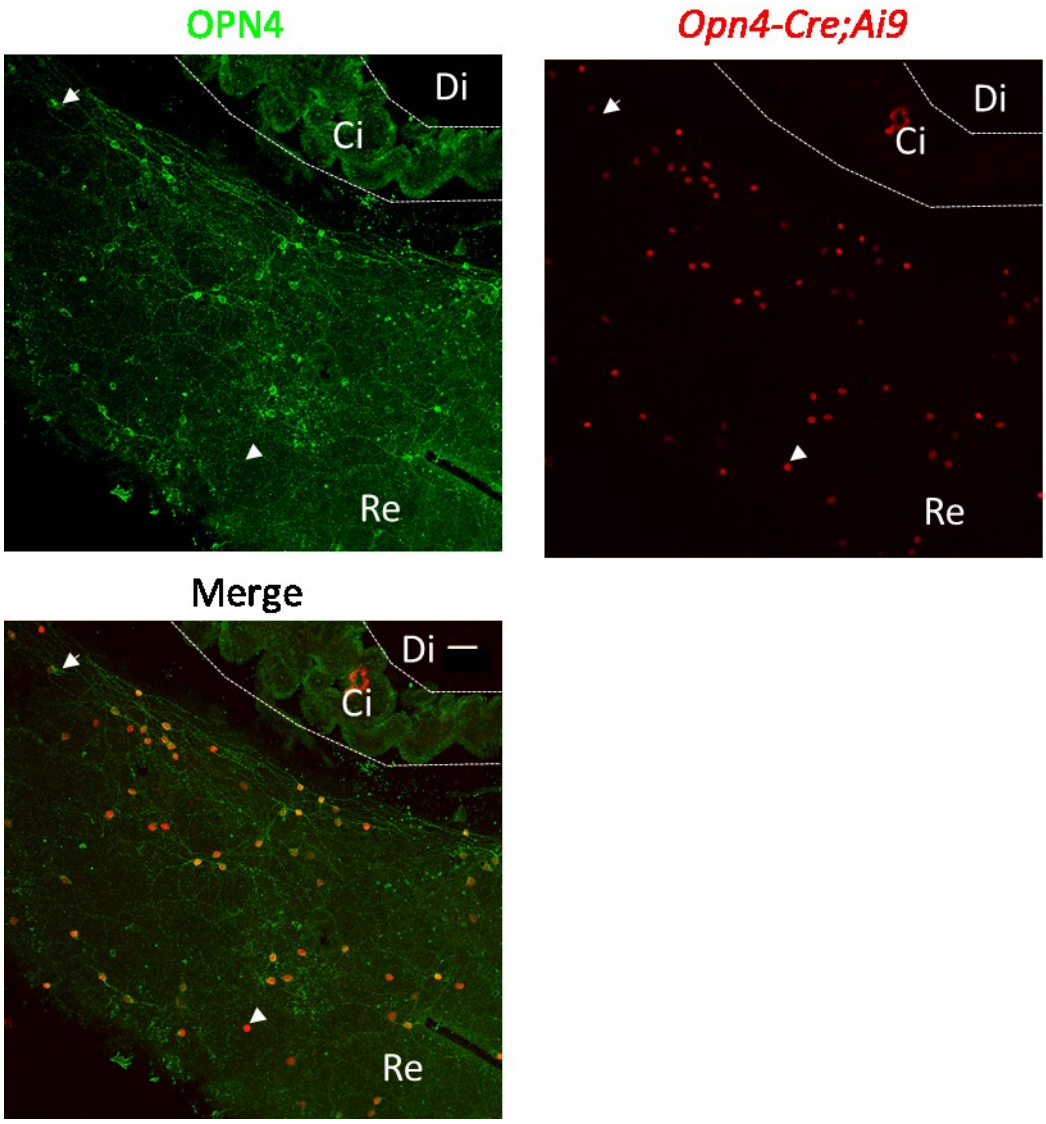

Figure 3-8 Specificity of the *Opn4-Cre* line in mouse retina. *Opn4-Cre;Ai9* retina immunostained for melanopsin (OPN4, green) to verify the specificity of the genetic-labeling. Red indicates fluorescence directly from tdTomato. In this

image, 86% of tdTomato-positive cells show melanopsin immunoreactivity (arrowhead indicates one example of tdTomato-positive but melanopsin-immunonegative cell). 96% of melanopsin-immunopositive cells were tdTomato labeled (arrow indicates one example of tdTomato-negative but melanopsin-immunopositive cell). Di: dilator; Ci: ciliary body; Re: Retina. Scale bar = 50μm.

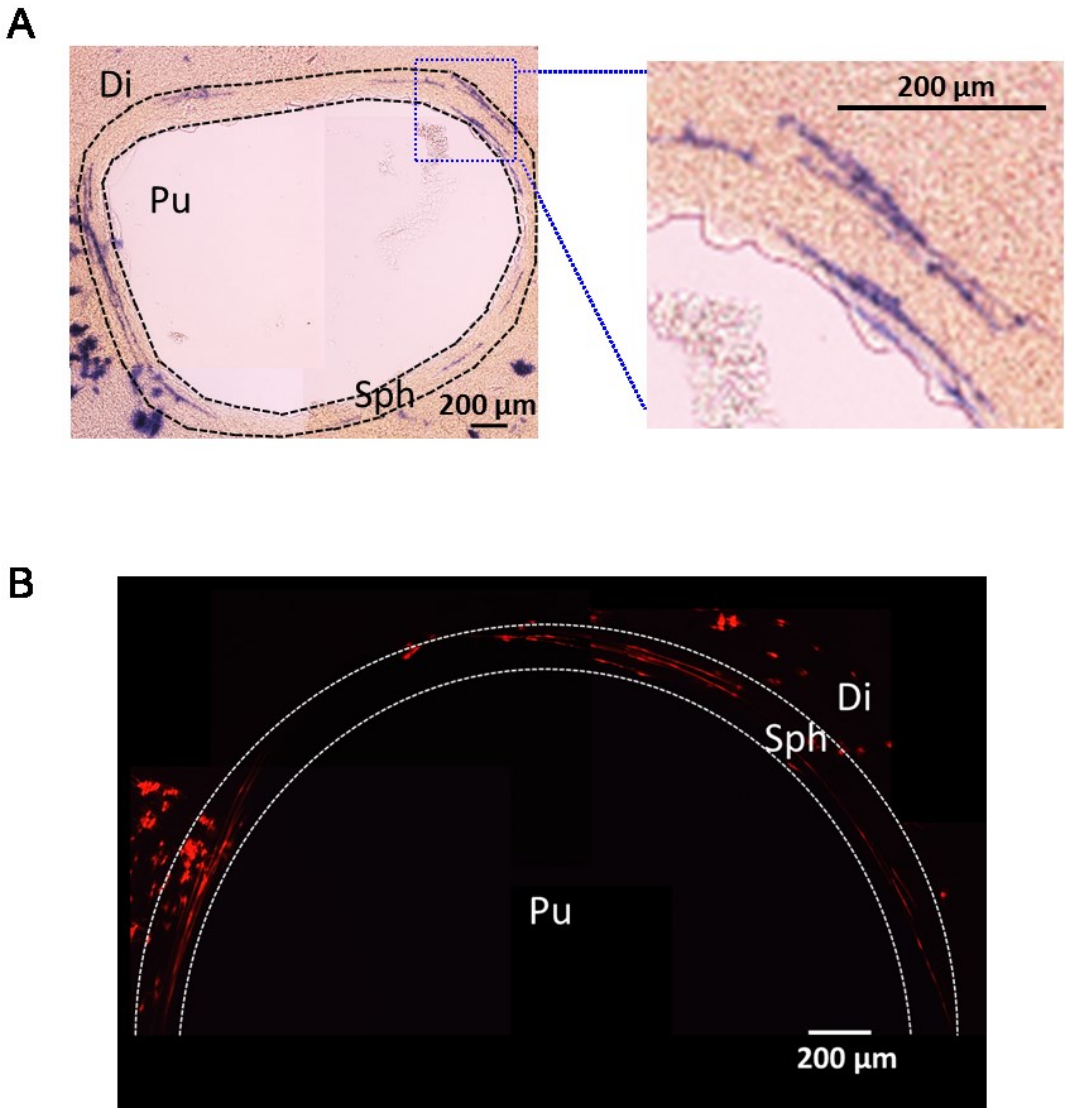

Figure 3-9 Melanopsin expression in mouse iris. A, iris isolated from *Opn4-Cre;R26iAP* animal. Melanopsin expression (purple) can be found in both sphincter muscle region and dilator muscle region. B, iris taken from *Opn4-Cre;Ai9* animal. TdTomato signal reflects the activity of melanopsin promotor. Pu: pupil; Sph: sphincter; Di: dilator.

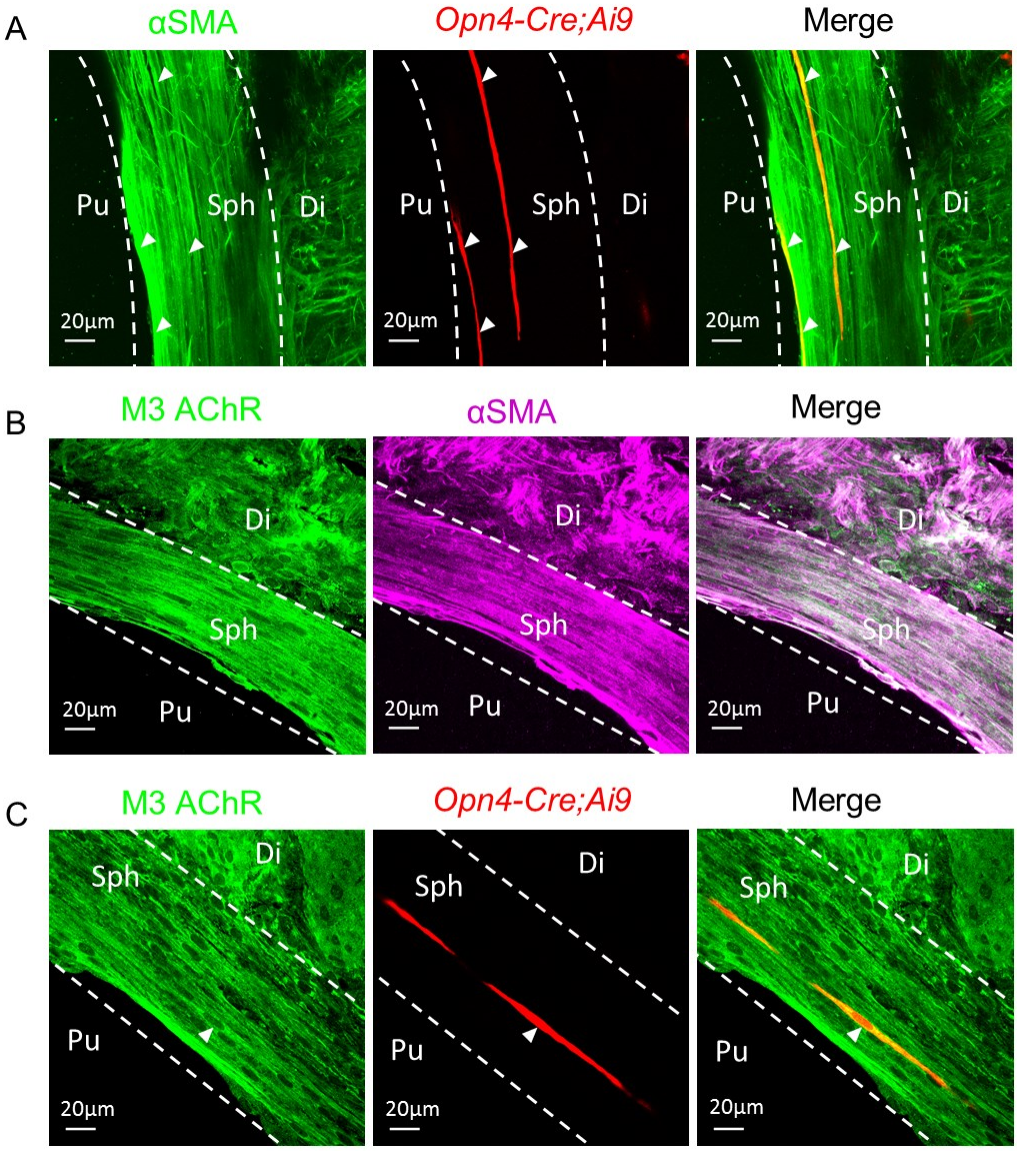

Figure 3-10 Melanopsin-expression in the mouse iris sphincter muscle cells.

A, whole-mount *Opn4-Cre;Ai9* (red) iris immunostained for α-smooth muscle actin (αSMA, green). Arrowhead marks tdTomato-positive sphincter muscle cells. B, whole-mount mouse iris immunostained for M3 muscarinic acetylcholine

receptor (M3 AChR, green) and αSMA (magenta) C, whole-mount *Opn4-Cre; Ai9* (red) iris immunostained for M3 AChR (green). Dotted lines demarcate the pupil (Pu), sphincter muscles (Sph) and dilator muscles (Di).

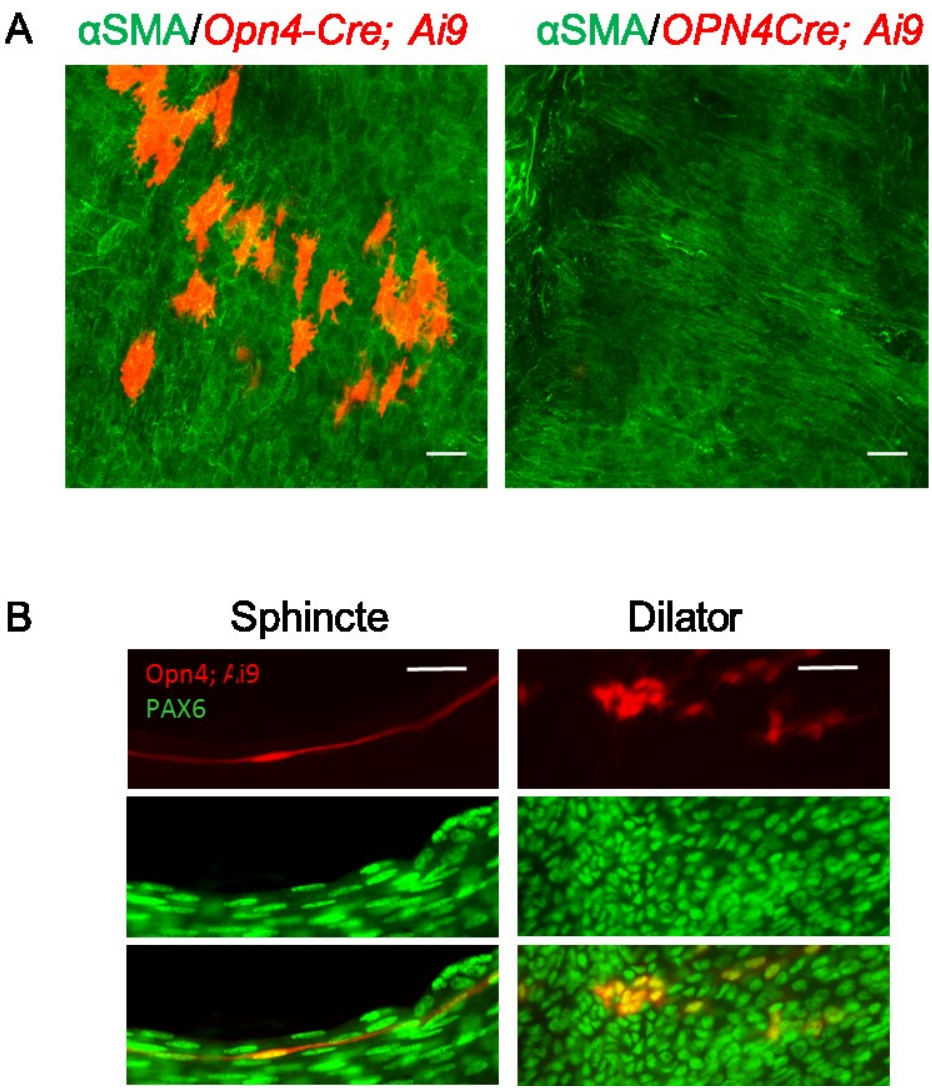

Figure 3-11 Melanopsin-expression in the dilator region of mouse iris. A, melanopsin-expressing cells in the mouse iris dilator region. The tdTomato-positive cells (in optical section on left) are not localized at the smooth muscle layer (in optical section on right). B, tdTomato-positive cells in both the sphincter and dilator muscle region are also immunopositive for PAX6 (green). Scale bars = 20 μm.

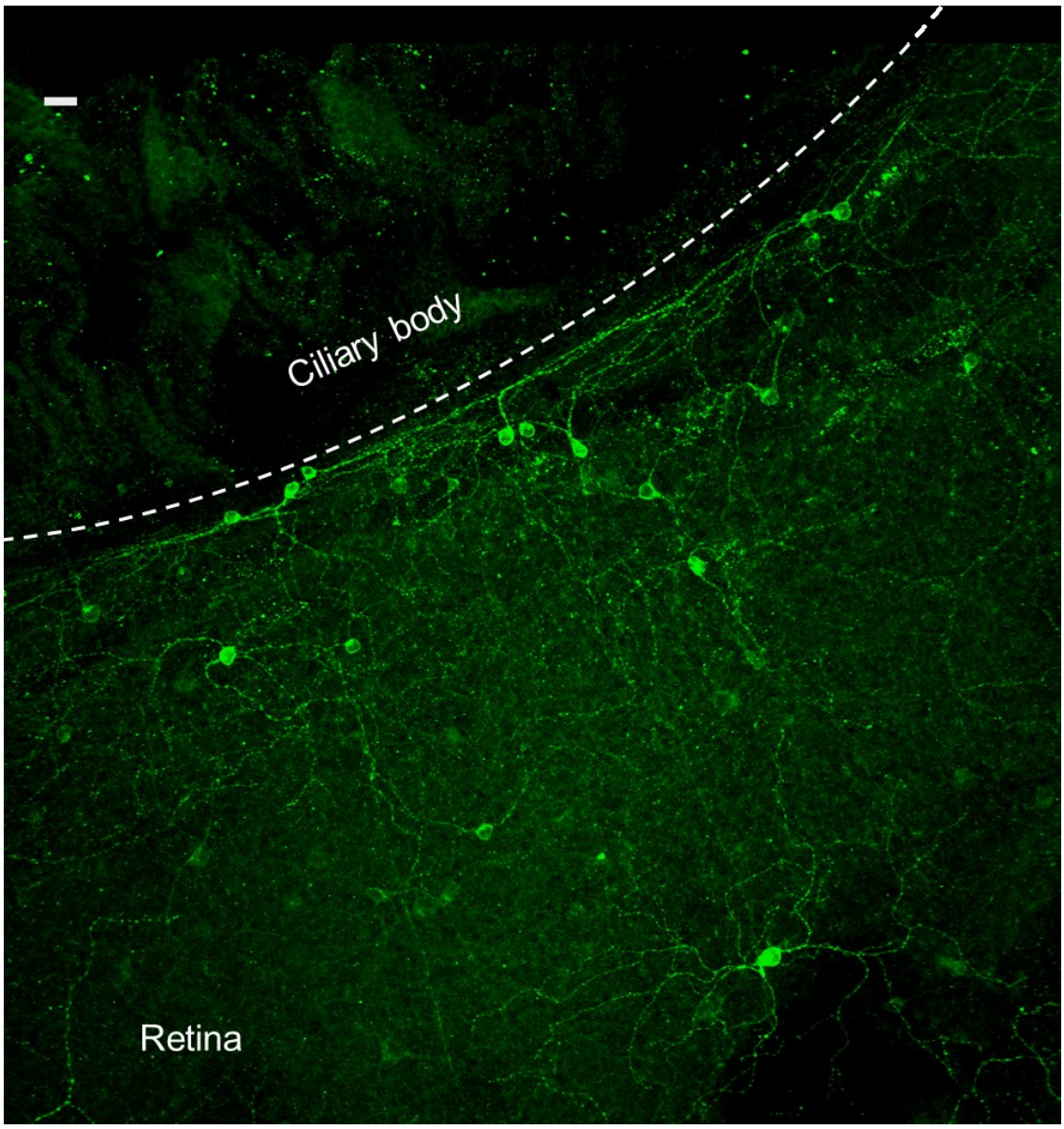

Figure 3-12 Melanopsin-positive plexus on the edge of retina. Whole mount mouse retina stained with anti-melanopsin antibody (green). No obvious fibers have extended into the ciliary body. Dotted line defines the junction between the retina and the ciliary body. Scale bar = 20μm.

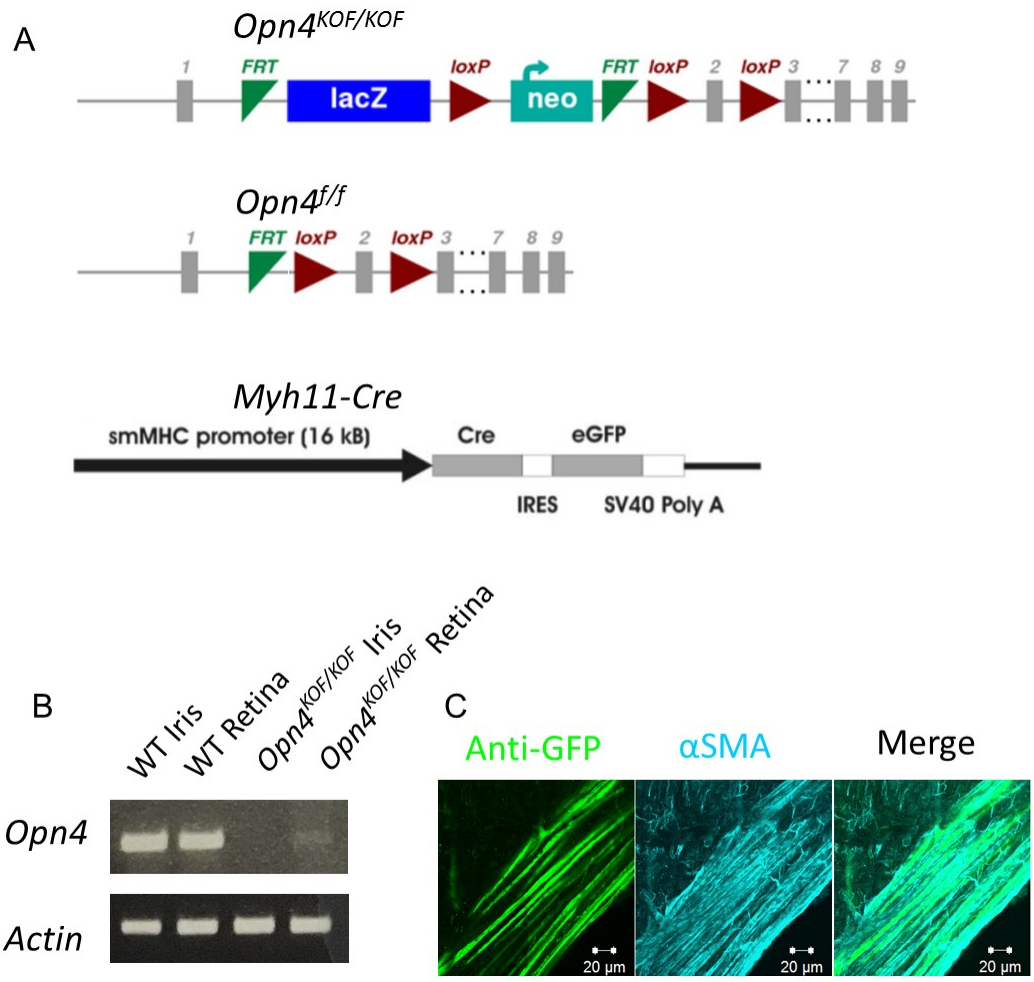

Figure 3-13 Tissue-specific ablation of melanopsin expression. A, the knockout-first allele (*Opn4*^{KOF}) is initially a non-expressive form, but can be converted to a conditional allele (*Opn4*^{f}) via flippase recombination. Tissue specific ablation of *Opn4* expression was achieved by crossing the *Opn4*$^{f/f}$ mouse line with *Myh11-Cre* line[95]. B, RT-PCR detection of *Opn4* transcripts in iris and

retina isolated from WT and *Opn4*$^{KOF/KOF}$ animals. C, expression of Cre in mouse iris smooth muscle (green: GFP; cyan: αSMA).

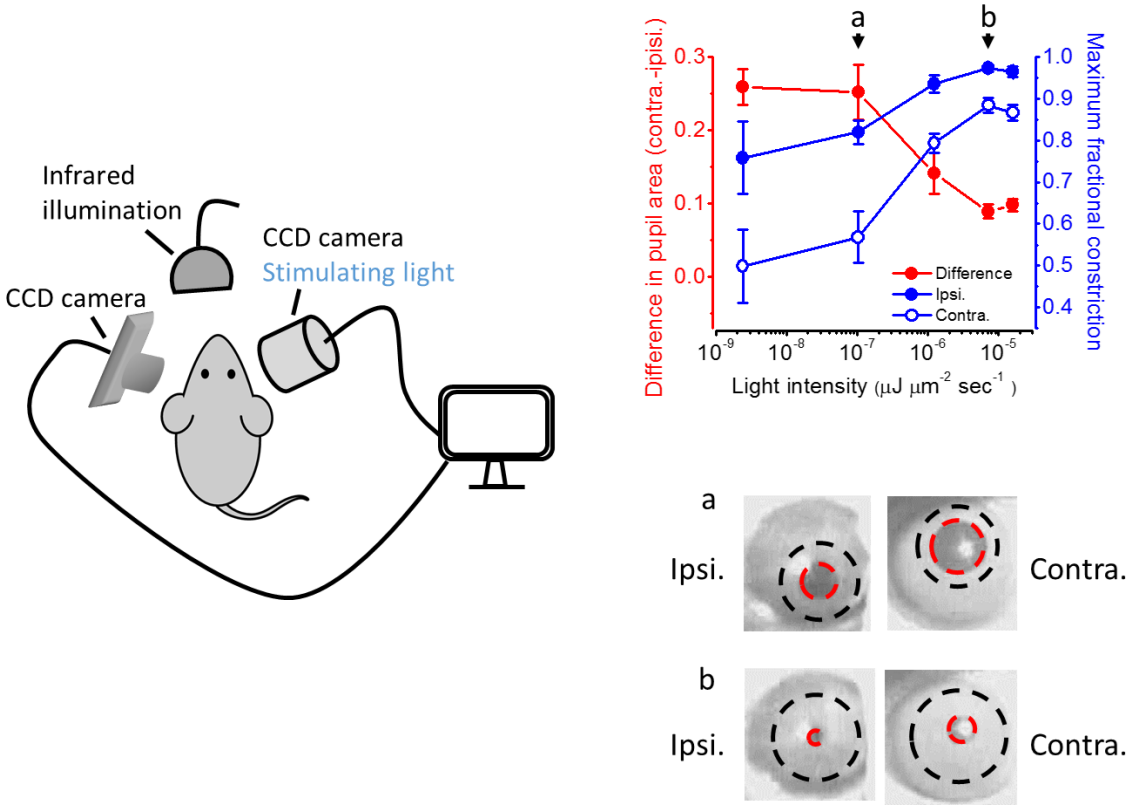

Figure 3-14 Simultaneous direct (ipsilateral) and consensual (contralateral) PLRs to unilateral illumination in WT mice. A, Schematic drawing of experimental paradigm. The pupil constriction (PLR) in both eyes was monitored with infrared CCD cameras, with only one side of the eye subjected to light sitmulation. B, PLR in ipsilateral, illuminated eye measured at the peak during the 30 sec stimulation period, with contralateral PLR measured simultaneously response (mean ± SD., 4 animals). a&b, examples of pupil area taken at the indicated light intensity. Illumination for 30sec with 505-nm LED light.

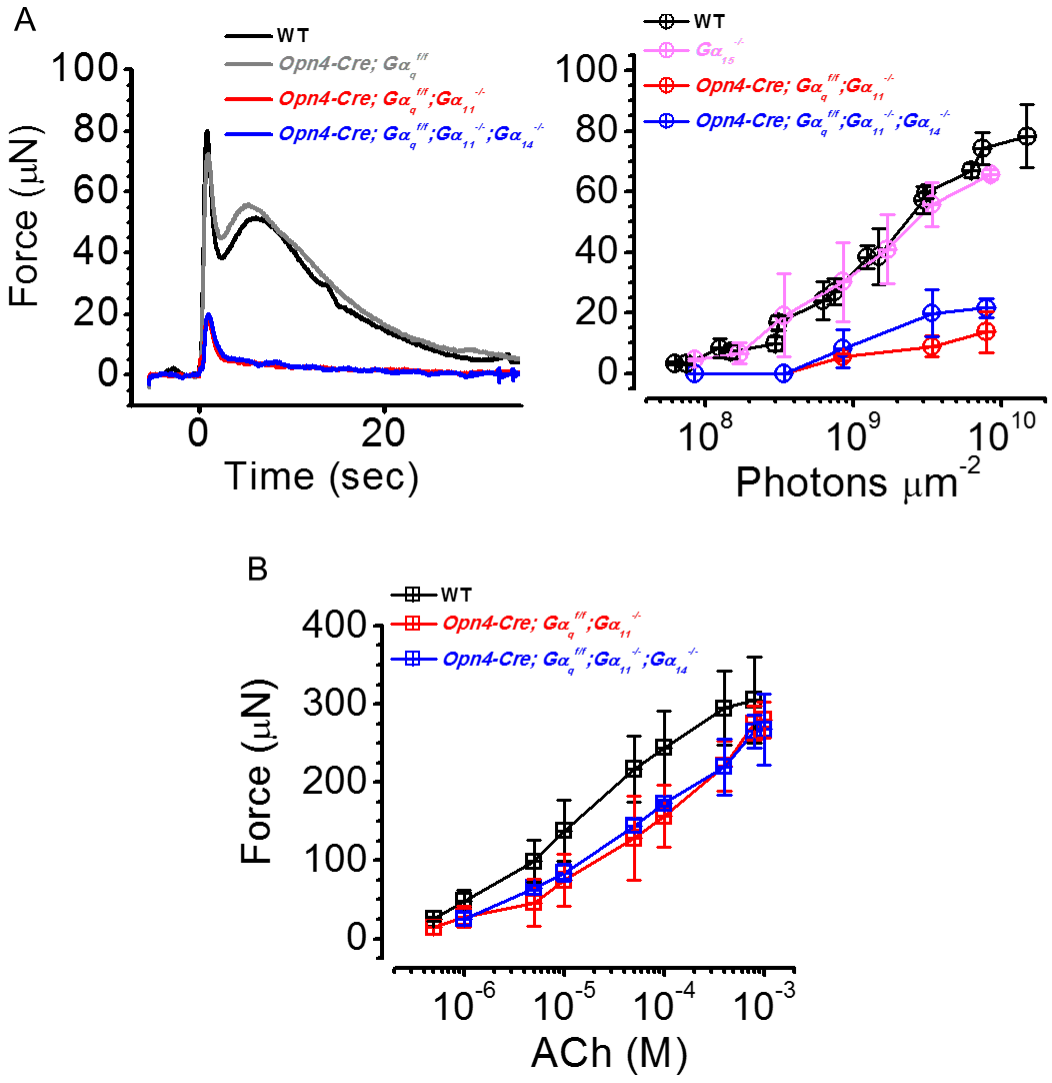

Figure 3-15 Signaling mechanism-mediating G-protein. A, left, sample responses of iris sphincter muscles from WT (black), *Opn4-Cre;*$G\alpha_q{}^{f/f}$ (gray), *Opn4-Cre;*$G\alpha_q{}^{f/f}$*;*$G\alpha_{11}{}^{-/-}$ (red) and *Opn4-Cre;*$G\alpha_q{}^{f/f}$*;*$G\alpha_{11}{}^{-/-}$*;*$G\alpha_{14}{}^{-/-}$ (blue) mice to a light flash (7.95×10^9 photons μm^{-2} at 436nm). Right, intensity-response relations

of *Gα$_{15}^{-/-}$* (magenta, mean ± SD, 3 muscles), *Opn4-Cre;Gα$_q^{f/f}$;Gα$_{11}^{-/-}$* (red, mean ± SD, 3 muscles) and *Opn4-Cre;Gα$_q^{f/f}$;Gα$_{11}^{-/-}$;Gα$_{14}^{-/-}$* muscles (blue, mean ± SD, 3 muscles). WT relation (black) from Fig. 2B is reproduced for comparison. B, ACh dose-response relations of WT (black, same as Fig. 2A*), Opn4-Cre;Gα$_q^{f/f}$;Gα$_{11}^{-/-}$* (red, mean ± SD, 3 muscles) and *Opn4-Cre;Gα$_q^{f/f}$;Gα$_{11}^{-/-}$;Gα$_{14}^{-/-}$* muscles (blue, mean ± SD, 2 muscles).

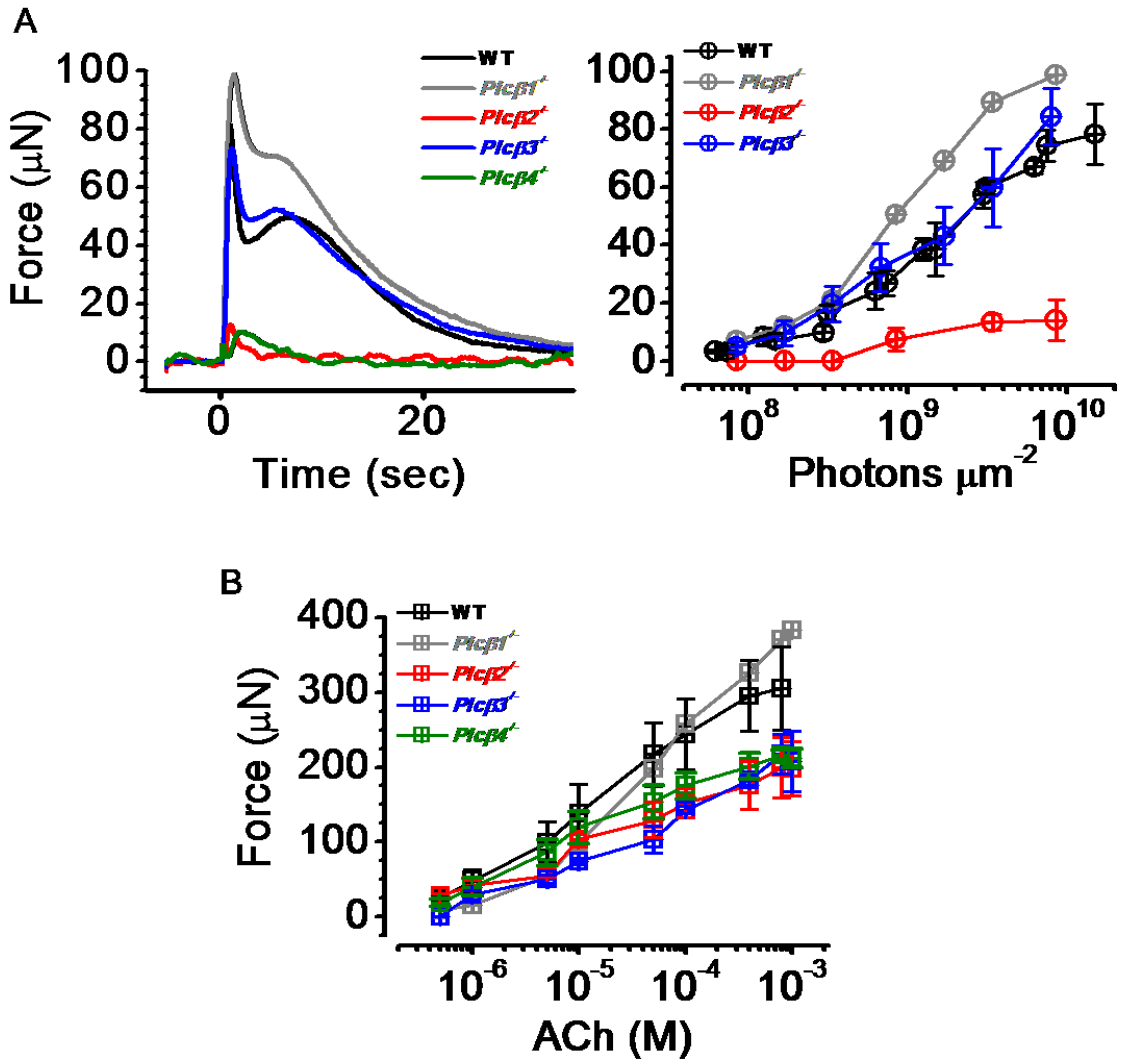

Figure 3-16 Signaling mechanism-PLCβ isoforms. A, left, sample responses of iris sphincter muscles from *Plcβ1*$^{-/-}$ (gray), *Plcβ2*$^{-/-}$ (red), *Plcβ3*$^{-/-}$ (blue) and *Plcβ4*$^{-/-}$ (green) mice to a light flash (8.52×10^{9} photons μm^{-2} at 436nm). Right, intensity-response relations for WT (black, same as Fig. 2B), *Plcβ1*$^{-/-}$ (gray, 1 muscle), *Plcβ2*$^{-/-}$ (red, mean ± SD, 4 muscles) and *Plcβ3*$^{-/-}$ (blue, mean ± SD, 3

muscles) muscles. The *Plcβ1*$^{-/-}$ muscle showed a stronger light response than WT possibly due to older age (2-year-old). B, ACh dose-response relation of WT (black, same as Fig. 2A), *Plcβ1*$^{-/-}$ (gray, 1 muscle, from 2-year-old animal), *Plcβ2*$^{-/-}$ (red, mean ± SD, 3 muscles), *Plcβ3*$^{-/-}$ (blue, mean ± SD, 3 muscles) and *Plcβ4*$^{-/-}$ (green, mean ± SD, 4 muscles) muscles.

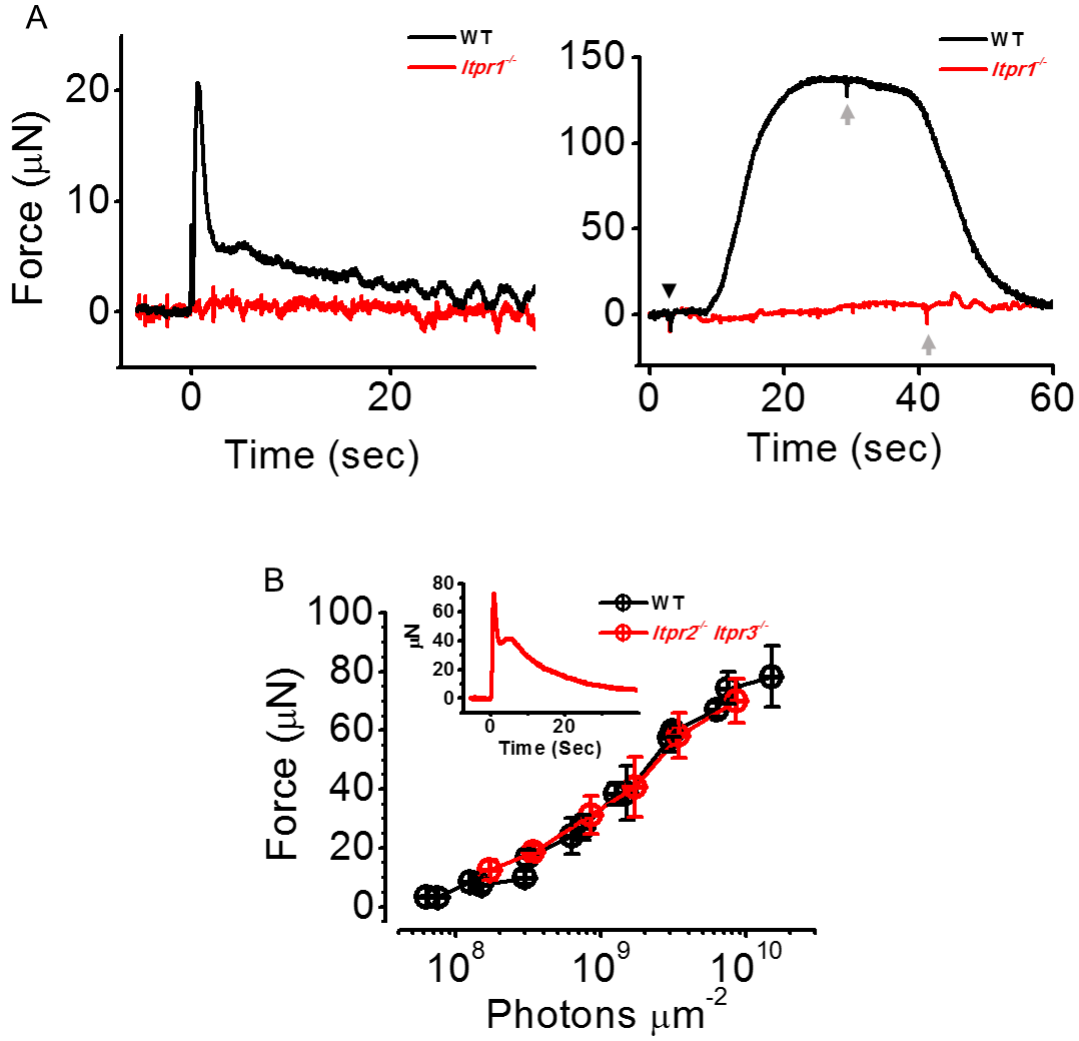

Figure 3-17 Signaling mechanism-IP3R isoform. A, sample responses of a WT (black, from P30 animal) and an *Itpr1*$^{-/-}$ (red, from P23 animal) sphincter muscle to light flash (8.52×10^{9} photons μm^{-2} at 436nm) and ACh (50 μM). On- and off-set of bath-applied ACh are indicated with black and gray arrow heads, respectively. B, intensity-response relations for WT (black, same as Fig. 1b) and

Itpr2$^{-/-}$;*Itpr3*$^{-/-}$ (red, mean ± SD, 3 muscles) muscles. Top inset shows sample response to *Itpr2*$^{-/-}$;*Itpr3*$^{-/-}$ to a light flash (7.95×10^{9} photons μm^{-2} at 436nm).

4. iPLR in Chicken

4.1 The photoreceptive role of cryptochrome in chicken iris

In 2004, Tu and colleagues reported that cryptochrome is involved in non-opsin-based photoreception in the chicken iris[56]. Their data showed that the isolated chicken iris constricted to light, with an action spectrum consistent with cryptochrome rather than other opsin pigments. Furthermore, they demonstrated that knockdown of cryptochrome decreased the photosensitivity of the iris. However, the cellular and molecular details underlying the cryptochrome-mediated photoreception remains to be determined.

To further investigate the photoreceptive role of vertebrate cryptochrome, we directly measured the force generated from the light-induced muscle contraction. The chicken iris sphincter muscle is a circumferential smooth muscle ring that controls the pupil size. We isolated the iris sphincter muscle from chicken embryo and, under infrared light, mounted the muscle ring horizontally on a fabricated force sensor. Upon illumination, the muscle contracts and the force generated can be recorded. This iris sphincter muscle preparation is stable and can give reproducible light responses for over 6 hours. Using this method, we generated the action spectrum of chicken iris by plotting the normalized sensitivity against wavelength (Fig.4-1). The sensitivity of the iris sphincter muscle kept on increasing as the wavelength decreased to blue/ultraviolet light, indeed very distinct from the behavior of melanopsin (with λ_{max} at 480 nm), but consistent with

that of cryptochrome[56]. The photosensitivity of chicken iris only appears in a short developmental time period (Fig. 4-2 and Ref. 55), possibly corresponding to the presence of photo-responsive smooth muscle cells in the chicken iris.

Specific pharmacological reagents were used to examine the signal transduction pathway of the chicken iris-based photoreceptor. Based on clues from the previous work on mouse iris[45], we first tested whether a PLCβ inhibitor would eliminate the chicken iris light response. Surprisingly, a substantial light response remained in the presence of the PLCβ inhibitor. This may be due to poor drug penetration into the tissue. The photoactivation of cryptochrome also depends on the flavin-based electron transport[57], so we next tested whether inhibition of the flavin redox reaction of cryptochrome would block the light response of chicken iris sphincter muscle. However, after half an hour of bath-applied flavin-specific redox inhibitor diphenyleneiodonium chloride (DPI), the iris still contracted upon light stimulation. These experiments need to be repeated and re-evaluated.

We also tried the gene-knockdown method using morpholino oligos. Depending on the sequence selected, morpholinos can be used to modify splicing events. In our case, we designed morpholino oligos that bind to the boundary between the second exon and the second intron, so that second exon of chicken *cryptochrome 1* and *2* (*cCry1* and *2*) would be eliminated, causing a frame shift that generates a premature stop codon in the third exon and thus a non-functional protein. We electroporated the isolated iris sphincter with control morpholinos and *cCry1* and *2*-specific morpholinos. The morpholinos have a

green-fluorescent-tag so that we could identify their expression in the iris sphincter muscle cells (Fig. 4-3A). After electroporation, the knockdown band of *cCry1* was detected (Fig. 4-3B). However, we were unable to detect any knockdown band of *cCry2* (Fig. 4-3B). Future experiments are required to optimize the knockdown efficiency.

Given that the lack of available genetic manipulations in non-model organisms such as chicken, we then decided to study the light-receptive role of chicken cryptochromes in heterologous system. Both *cCry1* and *cCry2* were cloned, and expressed HEK293 cells. Mouse cryptocrome 1 and 2 were also cloned and expressed for comparison. Anti-mouse CRY1 antibody could recognized the recombinant cCRY1, however, anti-mouse CRY2 antibody failed to recognize the recombinant cCRY2 (Fig. 4-4 and Fig. 4-5). Nevertheless, the availability of antibody for detecting cCRY1 lays the ground for investigating the expression pattern of cCRY1 in the chicken iris.

4.2 Summary and future direction

Our preliminarily data confirmed the findings, as reported by Tu and colleagues[56], that the isolated iris from chicken embryo constricts to light. The action spectrum of chicken iris showed a preferential wavelength of shorter than 436nm, deviated from that for melanopsin. The local photosensitivity in the chicken iris starts to appear around embryonic day 12, but decreased dramatically after hatch.

Knockdown of *cCry1* and *cCry2* resulted in a reduction in the photosensitivity of chicken iris[56], indicative of the involvement of cryptochromes in this process. It

remains to be determined that what percentage of the chicken sphincter muscle cells are photo-responsive, since the response amplitude to a step of bright light is about half of that induced by a step of ACh. Future experiments, such as immunolableing using anti-mCRY1 antibody, are needed to elucidate the localization of the light-responsive smooth muscles.

The general consensus is that the vertebrate cryptochromes are not light-receptive, and function as transcriptional repressors[57]. However, chicken cryptochrome might be one exception given its function in the local light reflex of the iris. The elucidation of its signaling mechanism will be of great impact on expanding our knowledge on the novel function of vertebrate crytochrome in photoreception.

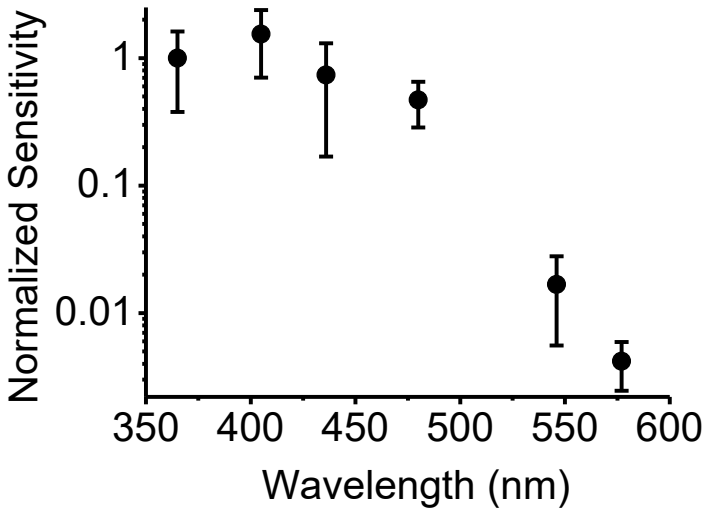

Figure 4-1 Action spectrum of chicken iris sphincter muscle. The action spectrum was generated by plotting the reciprocal quantal number required to produce a criterion tension level when the response amplitude is proportional to the number of incidental quanta. Sensitivity was normalized to the value at 365 nm (n=5, mean ± SD). Hg light with interference filters (365, 405, 436, 480, 546, 577 nm) were used.

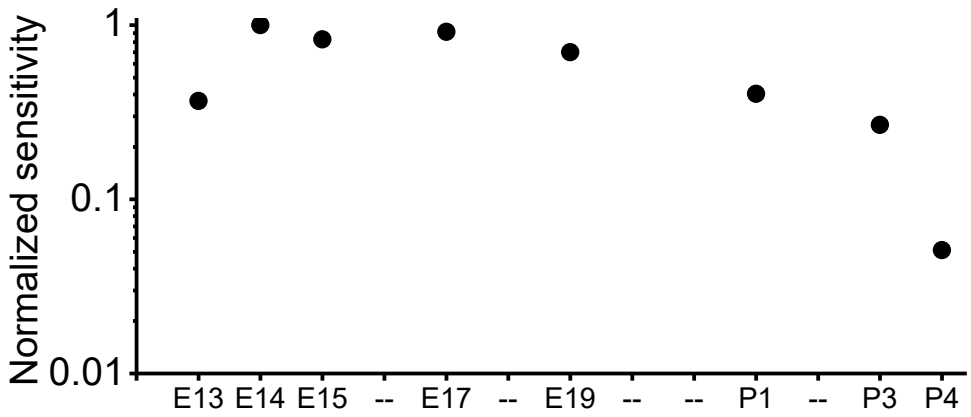

Figure 4-2 Age-dependency of the iPLR in chicken. Photosensitivity of iris isolated from chicken embryo (each data point represents mean from 2 to 4 irises) of different age was plotted. Sensitivity was normalized to the value at embryonic day 14 (E14).

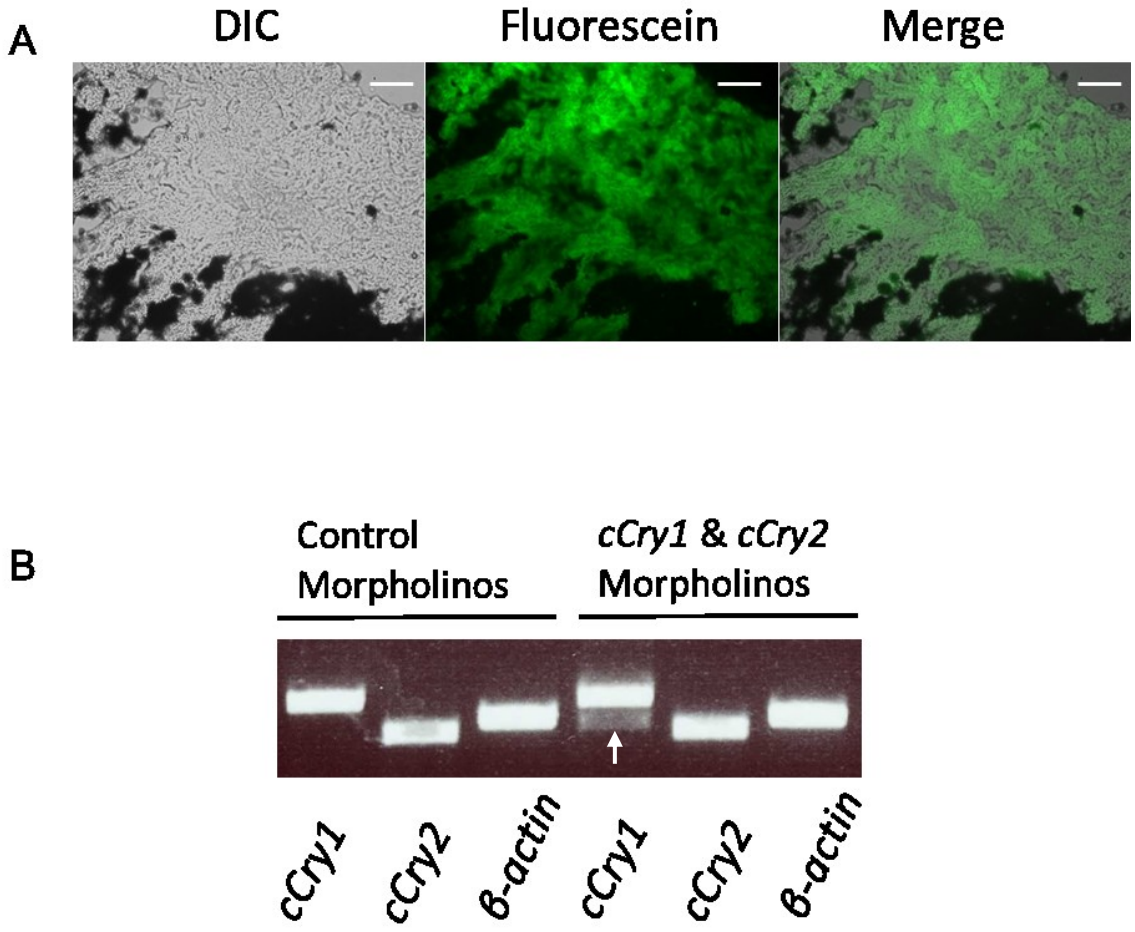

Figure 4-3 Knockdown of cryptochrome 1 and 2 in embryonic chicken iris. A, verification of the presence of morpholino oligos in chicken iris. After electroporation, fluorescent signal (shown in green) was detected in E14 embryonic chicken iris tissue. Scale bar = 20 μm. B, RT-PCR of chicken *β-actin*, *Cry1*, *Cry2*, and in E14 embryonic chicken iris tissue. Knockdown band for Cry1 was detected (marked with the arrow).

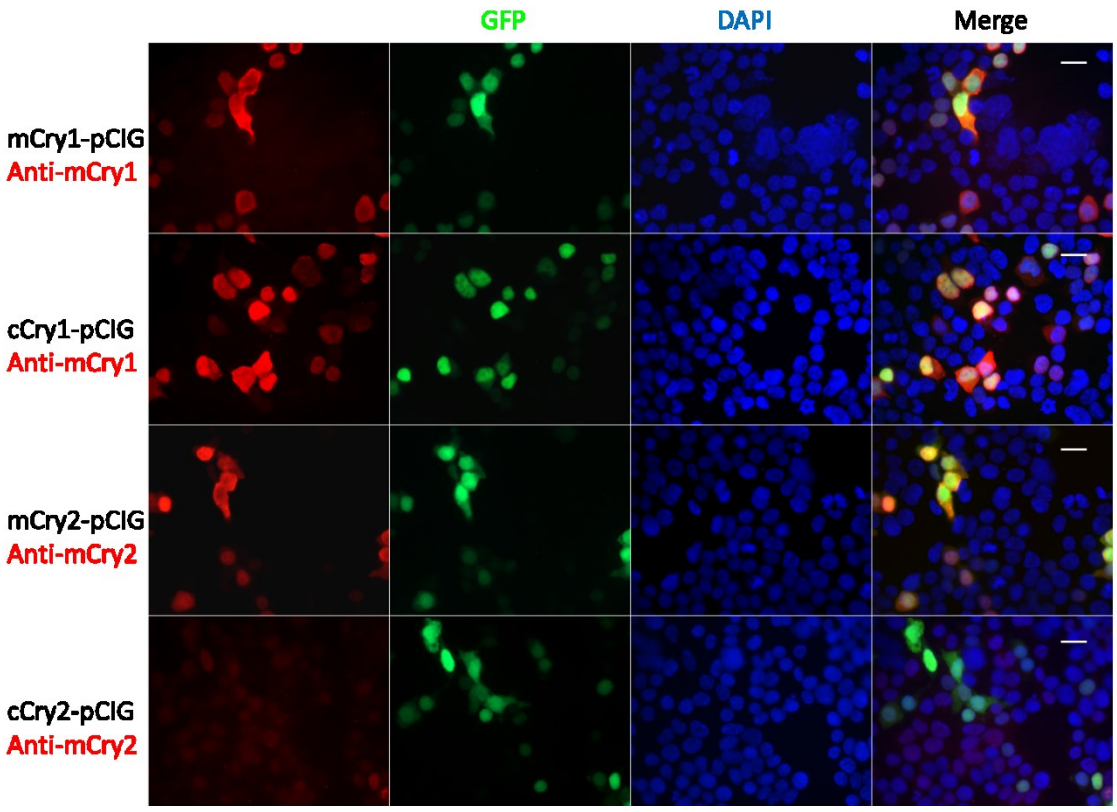

Figure 4-4 Heterologous expression of *cCry1*, *cCry2*, *mCry1*, and *mCry2* in HEK293 cells. Expression of recombinant *cCry1*, *cCry2*, *mCry1*, and *mCry2* was detected with anti-mouse CRY1 and CRY2 antibodies (red). Cells transfected with the recombinant vector also expressed GFP (green). Total amount of cells were visualized with nuclear staining (blue). Scale bar = 20 μm.

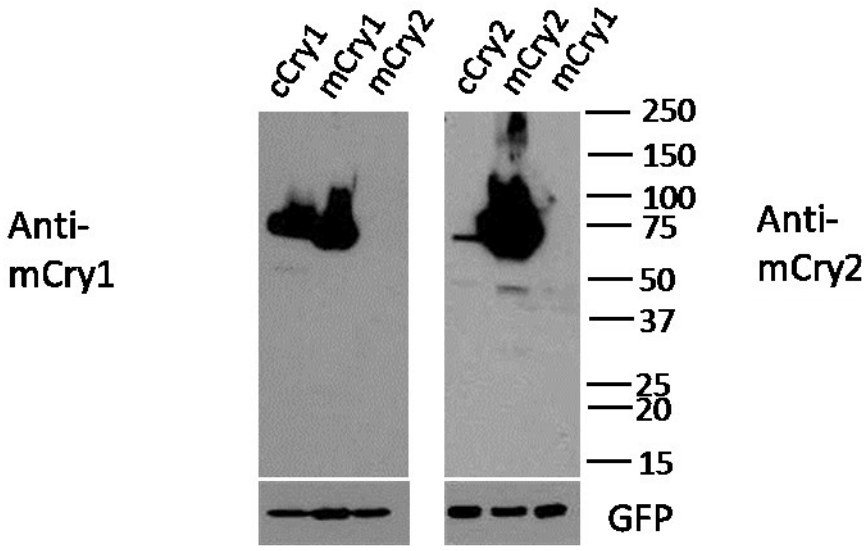

Figure 4-5 Western blotting of recombinant cCRY1, cCRY2, mCRY1, and mCRY2. Left, immunoblot using anti-mCRY1 antibody to detect recombinant mCRY1 and cCRY1. Left, immunoblot using anti-mCRY2 antibody to detect recombinant mCRY2 and cCRY2. GFP was used as loading control.

Attachment-Sequence of *floxed-Opn4* allele

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  ORGANISM  Mus musculus
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CURRICULUM VITAE FOR Ph.D. CANDIDATES

Johns Hopkins University School of Medicine

Qian Wang
Nov 14th, 2016

Educational History:

Ph.D. expected	2016	Program in Biochemistry, Cellular and Molecular Biology Mentor: King-Wai Yau Ph.D.	Johns Hopkins University School of Medicine
M.S.	2011	Program in Plant, Insect and Microbial Sciences Mentor: Qisheng Song Ph.D.	University of Missouri
B.S.	2008	Program in Biological Sciences	Shandong University

Other Professional Experiences:

Research Rotation	Sep-Dec 2011	Laboratory of Duojia Pan	Johns Hopkins University School of Medicine (Present location: The University of Texas Southwestern Medical Center)
Research Rotation	Mar-May 2012	Laboratory of Peter Devreotes	Johns Hopkins University School of Medicine

Awards and Honors

2016 Nominee for the Chinese Government Award for Outstanding Self-Financed Student Abroad

2010 Winner for Entomological Society of America Student Competition for the President's Prize-Poster Presentation

2010 The Phillip & Ruth Stone Scholarship Award for the Outstanding Master's Student in Entomology, University of Missouri

2007 Distinguished Student Award, Shandong University

2007 President Scholarship Award, Shandong University

2005 and 2006 First-Class Student Scholarship, Shandong University

2004 Freshman Scholarship, Shandong University

Publications

An, S†, Dong, S†, **Wang, Q**†, Li, S, Gilbert, LI, Stanley, D, Song, Q. Insect Neuropeptide Bursicon Homodimers Induce Innate Immune and Stress Genes during Molting by Activating the NF-κB Transcription Factor Relish. *PloS one* (2012) 7(3): e34510. († Co-first authors) PMCID: PMC3314635

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Posters and Oral presentations

Biochemistry, Cellular and Molecular Biology (BCMB) Graduate Program Retreat, Johns Hopkins University School of Medicine, Oct 2015
Melanopsin Signaling in Mouse Iris (Oral Presentation)

Entomological Society of America 58th Annual Meeting
San Diego, California, Dec 2010
The Role of Bursicon in Larval Stage (Poster)

Life Sciences Week, University of Missouri, Columbia, Missouri, April 2010
Identification of a Novel Bursicon function in *Drosophila melanogaster* (Poster)

Entomological Society of America 57th Annual Meeting
Indianapolis, Indiana, Dec 2009
Does Bursicon Play a Role in *Drosophila* Larvae? (Poster)

Service and Leadership

2015-2016 Member of Career Forum for Chinese Student and Scholar, Johns Hopkins University School of Medicine